Effect of dietary omega-3 supplementation on plasma phospholipids, neutral lipids fatty acids and antioxidant status of pregnant women with gestational diabetes and their neonates

A dissertation submitted for the degree of

Doctor of Philosophy (PhD)

By

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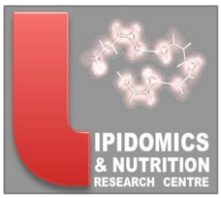

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Dedication

This dissertation is dedicated to **my late father** who always had confidence in me and offered me encouragement and support in all my endeavours; and I want to say (***O my Lord! Have mercy on them both, as they did care for me when I was young***) The Holy Qur'an, Chapter 17- Al-Isra': Verse 24.

Author's declaration

I certify that the thesis entitled: Effect of Dietary Omega-3 Supplementation on Plasma Phospholipids, Neutral Lipids Fatty Acids and Antioxidants Status of Pregnant Women with Gestational Diabetes and their Neonates, submitted for the degree of: Doctor of Philosophy, has not been previously submitted for another degree in this or any other educational institution.

This study was part of a large clinical trial conducted at Lipidomics and Nutrition Research Centre, London Metropolitan University, in collaboration with Newham University Hospital. I was responsible for the formulation and writing of the study protocol submitted to the Research Committee of the University, analyses of fatty acids and antioxidant vitamins (alpha-tocopherol, retinol and beta-carotene), laboratory data collation and evaluation, statistical analyses and interpretation of the results in consultation with Professor Ghebremeskel and Dr. Min and writing of the thesis.

Name: Sofia Eram

Signed……

Date……

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Abstract

Background: Gestational diabetes mellitus (GDM) has adverse effects on the level of docosahexaenoic acid (DHA) in phospholipids of maternal and cord red blood cells and cord plasma. This finding was of major concern because DHA is vital for maternal wellbeing and health, and for optimal development of foetal brain and retina. GDM is also associated with increased oxidative stress. There is controversy about omega-3 LCPUFA supplementation and oxidative damage. This ambiguity needs to be explored to reveal its role as modulator of oxidative stress in GDM.

Specific Aims: To investigate if (1) GDM adversely affects the plasma omega-3 and omega-6 long-chain polyunsaturated fatty acid (LCPUFA) levels in pregnant women. (2) High BMI is associated with adverse plasma fatty acid profile in GDM women. (3) Supplementation with DHA-enriched formula, enhances the level of the nutrient in the GDM women and their neonates. (4) Antioxidant vitamins status is enhanced by DHA-enriched supplementation in GDM women and their newborns.

Methods: Women with (n = 142; 72 active-group, 70 placebo) and without gestational diabetes (n = 28; 10 active-group, 18 placebo) were supplemented from the recruitment (at Newham University Hospital, London) until delivery. Both active- and placebo-groups received 2 capsules of either DHA-enriched formula or high oleic acid sunflower seed oil respectively. Each active supplement capsule contained 300 mg of DHA, 42 mg of eicosapentaenoic acid (EPA) and 8.4 mg of AA, and placebo 721 mg of oleic acid. Blood samples taken from the mothers at recruitment and delivery (maternal and cord) were analysed for plasma fatty acid composition and antioxidant vitamins levels.

Results: At recruitment, no significant difference was found in the DHA level in plasma phospholipids (CPG, 4.9% vs. 4.4%, $P > 0.05$) and neutral lipids (CE, 0.9% vs. 0.9%, $P > 0.05$), (TG, 0.9% vs. 0.9%, $P > 0.05$) between healthy pregnant and GDM women respectively. When categorized on the basis of their BMI, obese and over-weight GDM women had lower omega-3 (ALA, $P < 0.05$) and higher omega-6 PUFA (AA, $P < 0.05$) levels as compared to normal-weight GDM women. A total of 140 women completed the trial. GDM active-group compared with GDM placebo-group had significantly higher percentage of DHA in plasma CPG (4.4% vs. 3.7%, $P < 0.05$), CE (1.1% vs. 0.9%, $P < 0.05$), and TG (1.2% vs. 0.8%, $P < 0.05$) at delivery. There was no significant difference in the cord plasma [CPG (5.4% vs. 5.8%, $P > 0.05$), CE (1.1% vs. 1.0%, $P > 0.05$), TG (2.9% vs. 3.3%, $P > 0.05$)] DHA between GDM placebo and active-treatment groups. Though not significantly, the levels of vitamin A and β-carotene were reduced, however, the level of vitamin E was comparable between GDM and healthy pregnant women, at recruitment ($P > 0.05$). At delivery, no significant difference was found in maternal plasma vitamin A (21.1 μg/dl vs. 18.0 μg/dl, $P > 0.05$), vitamin E (1.4 mg/dl vs. 1.4 mg/dl, $P > 0.05$) and β-carotene (16.1 μg/dl vs. 11.1 μg/dl, $P > 0.05$) levels between GDM placebo- and active-treatment groups. Neonatal plasma antioxidant vitamins levels were also comparable between GDM active-treatment and placebo groups ($P > 0.05$).

Conclusion: The present study shows that the plasma DHA and AA levels are not compromised by gestational diabetes in pregnant women. It may be that the comparable plasma DHA and AA levels observed in the GDM women is linked to a failure to incorporate these fatty acids into the phospholipids of the red cell membrane and/or impaired placental transport. Moreover, the majority of samples were collected during the third trimester (between 28-32 weeks), so it is plausible that the duration of the diabetes was very short to produce an obvious adverse effect on the plasma DHA and AA levels. Additionally, this study shows that higher pre-pregnancy BMI is associated with higher n-6 PUFA and lower n-3 PUFA levels in GDM women. However, it is difficult to establish whether BMI causes adverse fatty acids profile, or whether the direction of this association is reversed.

This unique study also demonstrated that supplementation with a daily dose of DHA (600mg) from diagnosis until delivery was effective in enhancing the level of the nutrient in plasma of GDM women but not foetal. The inefficacy of the supplement to improve foetal status suggests that the transfer of DHA across the placenta may be impaired in the GDM women. This finding has implications for the management of neonates born to GDM women because they are born with a lower level of DHA and the condition is considered to be linked with a risk of neuro-developmental deficit. We suggest that the provision of a DHA supplement should be integrated with the antenatal care of pregnant women with gestational diabetes to optimize foetal development and avert maternal DHA depletion in

pregnancy. Also, the babies of the GDM women, particularly those not sucking mother's milk, similar to those who born prematurely require formula milk containing a higher level of DHA.

This study demonstrates that DHA-enriched supplement did not improve yet not deteriorate the antioxidant vitamins status in GDM women. This may be because of small dose and short duration of supplementation. We can allude that the moderate amounts of omega-3 LCPUFA in dietary intake for longer duration may reduce the incidence and complications associated with oxidative stress in diabetic pregnancy.

Original Publications

Min Y, Djahanbakhch O, Hutchinson J, Bhullar AS, Raveendran M, Hallot A, **Eram S,** Namugere I, Nateghian S Ghebremeskel K (2014). Effect of docosahexaenoic acid-enriched fish oil supplementation in pregnant women with Type 2 diabetes on membrane fatty acids and fetal body composition-double--blinded randomized placebo-controlled trial. *Diabetic Medicine*; **31**(11): 1331-40.

Min Y, Djahanbakhch O, Hutchinson J, **Eram S**, Bhullar AS, Namugere I, Ghebremeskel K (2016). Efficacy of docosahexaenoic acid-enriched formula to enhance maternal and fetal blood docosahexaenoic acid levels: randomized double-blinded placebo-controlled trial of pregnant women with gestational diabetes. *Clinical Nutrition*; **35**(3): 608-14.

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Abbreviations

AA	Arachidonic acid
ACOG	American Congress of Obstetricians & Gynecology
ACP	Acyl carrier protein
ADA	American Diabetes Association
ADIPS	Australasian Diabetes in Pregnancy Society
AI	Adequate intake
AIDS	Acquired Immunodeficiency Syndrome
ALA	Alpha linolenic acid
ATP	Adenosine triphosphate
ATP-III	Adult Treatment Panel III
BHT	Butylated Hydroxy Toluene
BMI	Body Mass Index
CDA	Canadian Diabetes Association
CE	Cholesterol Ester
CHDs	Coronary heart diseases
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CPG	Choline phosphoglycerides
DHA	Docosahexaenoic acid
DM	Diabetes Mellitus
DPA	Docosapentaenoic acid
EASD	European Association for the Study of Diabetes
ECD	Electron Capture Detector
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic Acid
HLA	Human Leukocyte Antigen
EFAD	Essential fatty acids deficiency
EFAs	Essential fatty acids
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
EPG	Ethanolamine phosphoglycerides
FABPs	Fatty acid binding proteins

FAME	Fatty Acid Methyl Ester
FIDs	Flame Ionization Detectors
GC	Gas Chromatography
GDM	Gestational Diabetes Mellitus
GLC	Gas Liquid Chromatography
GLUT	Glucose Transporters
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSLs	Glycosphingolipids
GSSG-Red	Glutathione reductase
HAPO	Hyperglycemia and Adverse Pregnancy Outcomes
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HSD	Tukey's honest significant difference test
IADPSG	International Association of the Diabetes and Pregnancy Study Groups
IDF	International Diabetes Federation
IPG	Inositol Phosphoglyceride
ISSFAL	International Society for the Study of Fatty Acids and Lipids
IUPAC	The International Union of Pure and Applied Chemistry
LA	Linoleic acid
LCPUFAs	Long chain polyunsaturated fatty acids
LDL	Low Density Lipo-proteins
LGA	Large for gestational age
LNRC	Lipidomics and Nutrition Research Centre
MNT	Medical and nutritional therapy
MS	Metabolic syndrome
MUFAs	Monounsaturated fatty acids
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear Factor kappa B
NICE	National Institute for Clinical Excellence

NICU	Neonatal intensive care units
NL	Neutral Lipids
NO	Nitric oxide
NPH	Normal Pressure Hydrocephalus
OFN	Oxygen free Nitrogen
OGTT	Oral glucose tolerance test
PA	Phasphatidic acid
PCOS	Polycystic Ovarian Syndrome
PDA	Photodiode Array
PG	Phosphatidyglycerol
PL	Phospholipids
PPARγ	Peroxisome proliferator-activated receptor gamma
PUFAs	Polyunsaturated fatty acids
RCS	Reactive chloride species
RDA	Recommended dietary allowance
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCOT	Support coated open tubular
SFAs	Saturated fatty acids
SMBG	Self-monitoring of blood glucose
SOD	Super Oxide Dismutase
SPG	Serine phosphoglycerides
SPM	Sphingomyelin
TBARSs	Thiobarbituric Acid-Reactive Substances
TCD	Thermal Conductivity Detector
TG	Triglyceride
TLC	Thin Layer Chromatography
TRANS-FA	Trans fatty acids
UV	Ultraviolet
VLDL	Very low Density Lipo-proteins
WCOT	Wall coated open tubular
WHO	World Health Organization

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CHAPTER 1

Introduction----------(1-58)

1.1 Study overview

The significance of essential fatty acids (EFAs) and their long-chain polyunsaturated fatty acids (LCPUFAs) in human development discovered its origin in 1929 when Burr and Burr explained linoleic acid's essentiality. They observed that the deficiency of linoleic acid in laboratory animals led to the symptoms of dermatitis, infertility and growth retardation, after its removal from their diets. The cure of these symptoms by vegetable oil supplementation further supported the essentiality of certain fatty acids as dietary constituents (Lauritzen et al. 2001). Hansen and co-workers studied the fatty acid deficiency in humans (Innis 2000). The essentiality of omega-3 fatty acids in "new-born" nutrition was not well-recognised until demonstrated by Holman in 1982. According to him, lack of omega-3 fatty acids are linked with clinical anomalies, such as weakness, paresthesia, impaired vision and inability to walk in a 6-years old child (Hadders-Algra 2004). At the same time, evidence was collected that omega-3 deficient diets induce some visual deformities in subhuman primates (Groen et al. 2005).

In last ten years or so, scientists have recognised the significance of omega-3 LCPUFAs during gestation and early life of child's development. It has been explained that sufficient levels of omega-3 fatty acids boost the growth of the foetal and infant brain and may increase cognitive development. Omega-3 fatty acids are also essential for maternal wellbeing. Recent research shows that omega-3 fatty acid intake by pregnant women decreases the risk of infection, allergies and asthma in infants (Velzing-Aarts et al. 2001). Omega-3 fatty acids, not only provide shelter from cardiovascular disease (Kris-Etherton et al. 2003), lower triglyceride levels (Von-Schacky and Harris 2007) but also have a significant effect on maternal health during pregnancy (Szajewska et al. 2006; Greenberg et al. 2008). They also may protect from preeclampsia (Kemse et al. 2014), reduce the possibility of preterm labour (Saccone and Berghella 2015) and the risk for perinatal and postpartum depression (Sallis et al. 2014).

Currently, considerable developments are under way on the understanding of physiological functions of LCPUFAs in animals and humans and their role in curing many diseases. The rectification of maternal and feotal plasma omega-3 LCPUFAs levels particularly Docosahexaenoic acid (DHA) by supplementing the gestational

diabetic women with DHA-enriched formula is the primary focus of this thesis. In addition, the effect of DHA-enriched supplementation on antioxidant vitamins status in GDM women and their babies has also been investigated in this study.

1.2 Fatty acids

1.2.1 Introduction

Fatty acids are aliphatic mono-carboxylic acids occurring mainly in natural triglycerides. All fatty acids have a single carboxyl group at the end of a hydrocarbon chain that ranges in length mostly from 4 to 20. With some exceptions, they contain an even number of carbon atoms, because, in the human body, fatty acid synthesis involves the addition of 2 carbon atoms. Hence, it is said that fatty acids are made up of carbon chains with a methyl group at one end (designated Omega, ω) and a carboxyl group attaches at the other end of the molecule (**Figure 1.1**). Alpha (α) carbon is the carbon atom that lies next to the carboxyl group while the beta (β) carbon lies next to α-carbon.

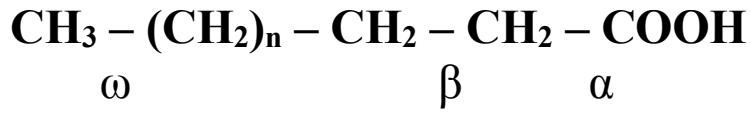

Figure 1.1. Essential features of a fatty acid structure.

Fatty acids either free or as a part of complex lipids, play some vital roles in metabolism, acting as major metabolic fuel (storage and transport of energy), as essential components of all membranes and as gene regulators. Fatty acids are one of the substrates involved in energy production by β-oxidation to maintain metabolic homeostasis (IUPAC 2007).

1.2.2 Nomenclature

There are several systems of nomenclature for fatty acids. The most common are described underneath (IUPAC 2007).

1.2.2.1 Trivial/Common nomenclature: It is the most common naming system used in literature, which includes non-systematic and historical names. The most frequently occurring fatty acids also have trivial names along with their systematic names. These names mostly do not follow any pattern, but they are often unambiguous and concise. Common names usually reflect the source or the name of the discoverer. For example, palmitic acid from palm oil or mead acid named so after James Mead.

1.2.2.2 Systematic nomenclature: Systematic or IUPAC (The International Union of Pure and Applied Chemistry) names are derived so after the standard rules of IUPAC made for the nomenclature of organic chemistry, published in 1979 (Rigaudy and Klesney 1979), accompanied by a recommendation published particularly for lipids in 1977 (IUPAC-IUB 1977). Counting begins with the carbon of carboxylic acid end to the methyl end. Double bonds are labelled with *cis*- *trans*- notation wherever found appropriate. This notation is mostly more verbose than common nomenclature, but technically, it has the advantage of being more clear and descriptive. For example, 9-octadecenoic acid.

1.2.2.3 Delta nomenclature: In Delta nomenclature, Δ^x indicates the position of double bonds with reference to the carboxyl acid end (IUPAC-IUB 1977). A *cis*- or *trans*- prefix precedes each double bond that indicates the conformation of the molecule around the bond. For example, "*cis*-Δ^9, *cis*-Δ^{12} octadecadienoic acid" is designated as linoleic acid. This nomenclature is less verbose than systematic nomenclature but technically is not more clear or descriptive.

1.2.2.4 n-nomenclature: n-x or omega-x nomenclature, both specifies names for individual compounds and classes them by their likely biosynthetic features in animals. A double bond is positioned on the X^{th} carbon-carbon bond, counting from the terminal methyl carbon (termed as *n* or ω) toward the carbonyl carbon. For example, α-linolenic acid is classified as an n-3 or omega-3 fatty acid, so its biosynthetic pathway is expected to be alike with other compounds of this type. In nutritional literature, the notation ω-*x*, omega-*x*, or "omega" is commonly popular, but in technical documents, IUPAC has disapproved it for *n-x* notation. For example, *cis*-Δ^9, *cis*-Δ^{12} octadecadienoic acid is notated as 18:2n-6.

1.2.3 Classification of fatty acids

The predominant fatty acids are the straight chain and can be categorised as saturated and unsaturated fatty acids. This classification is made on the presence of double bonds and its number in their hydrocarbon chain (DeFilippis and Sperling 2006).

1.2.3.1 Saturated fatty acids

Saturated fatty acids (SFAs) have no double bond in their hydrocarbon chain. Therefore, they are saturated with hydrogen (double bonds decrease the number of hydrogens on each carbon). SFAs consist of the only single bond. Thus, each carbon atom in the hydrocarbon chain has two hydrogens (except for the ω carbon at the methyl end which has three hydrogens). Most of the SFAs occurring in nature are unbranched and have even numbers of carbon atoms. They have a general formula R-COOH, in which R group is a straight chain hydrocarbon of the form $CH_3(CH_2)_X$. SFAs are very stable as they are least reactive chemically. The melting point of SFAs increases with chain length. The single bonds of the SFAs give them a fully extended and relatively linear structure so that these types of fatty acid can be packed very closely with almost crystalline structure. The longer chain fatty acids are solid at room temperature.

Saturated fatty acids are further classified into three subgroups; short, medium and long. Short chain fatty acids consist of less than 8-carbon atoms. The important members of this group are butyric (4:0) and caproic (6:0) acids (occurring mainly in milk fat). The second subgroup, medium chain fatty acids are fatty acids with the carbon atoms ranging from 8-14. Capric acid (10:0), lauric (12:0) and myristic acid (14:0) are members of this group. Among them, myristic acid (14:0) has a widespread occurrence, but occasionally as a major component. The long chain fatty acids are the fatty acids with 16 carbon atoms or more. Palmitic acid (16:0) is the most commonly occurring saturated fatty acid in animals, plants and microorganisms. Stearic acid (18:0) is also a vital member of this group and is the main fatty acid in animals and some fungi though a minor constituent in most plants (Ratnayake 2008; Olsen et al. 2009), (**Figure 1.2**).

$CH_3 - (CH_2)_5 - CH_2 - CH_2 - CH_2 - CH_2 - CH_2 - (CH_2)_3 - CH_2 - COOH$

Figure 1.2. Saturated fatty acid, Palmitic Acid (C16:0).

1.2.3.2 Unsaturated fatty acids

Unsaturated fatty acids consist of a hydrocarbon chain with single or more double bonds. Monounsaturated fatty acids (MUFAs, monoenoic) have one double bond in their hydrocarbon chain. While, polyunsaturated fatty acids (PUFAs, polyenoic) have two or more double bonds usually separated by a single methylene group in a carbon skeleton (De-fillipis and Sperling 2006).

1.2.3.2.1 Monounsaturated fatty acids

MUFAs contain a single carbon-carbon double bond located in different positions. The presence of a single double-bond in the structure offers two possible patterns; *cis* and *trans*. *Cis* configuration results in a non-straight structure, which confers more fluidity to the membranes as compared to the *trans* configuration. *Trans* fatty acids are, in fact, straight chains with properties similar to saturated fatty acids and they increase membrane rigidity. Many fatty acids in the *trans* structure are not present in nature and may be produced by hydrogenation of unsaturated oils during industrial processing and in the digestive tract of ruminants. *Cis* configuration gives a bend in the linearity of the hydrocarbon chain making these fatty acids not to be packed as tightly as the saturated fatty acids. The thermodynamical stability of *cis* fatty acids is less than the *trans* forms. Therefore, they have lower melting points as compared to the *trans* fatty acids or their saturated counterparts. The melting point of unsaturated fatty acids decreases with increasing unsaturation (Olafsdottir et al. 2010; Szajewska et al. 2012).

The most common MUFAs have a chain length of 16-22 with a double bond in the *cis* configuration. All naturally occurring unsaturated fatty acids of mammals (especially in the cell membranes) are of the *cis* configuration (Shantha and Napolitano 1992; Weijers 2012). The double bond is most likely located at the Δ^9 position. Oleic acid (*cis*-9-octadecenoic, 18:1n-9) is the commonest *cis*-MUFA, and it is also the most widely distributed amongst all natural lipids. Palmitoleic acid (16:1n-7) is also present widely in plants, animals and microorganisms and is one of the major components in some seed oils (Rustan and Drevon 2005), (**Figure 1.3**).

$$CH_3 - (CH_2)_5 - CH_2 - CH_2 - CH = CH - CH_2 - (CH_2)_5 - CH_2 - COOH$$

Figure 1.3. Monounsaturated fatty acid, Oleic Acid (C18:1n-9).

1.2.3.2.2 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) consist of two or more double bonds. The double bonds can be uninterrupted (allenic), one-methylene-interrupted or polymethylene-interrupted. Most animal cells have one-methylene-interrupted double bonds. In PUFAs, the presence of several double bonds lowers their melting point compared to SFAs of the same length and produces extremely flexible molecules. Therefore, high "fluidity" of the cell membrane is usually associated with the presence of increased number of unsaturated fatty acids (Gunstone 1999; Ruxton et al. 2004).

The two biologically important subclasses of PUFA are n-3 (ω-3) and n-6 (ω-6) fatty acids. These fatty acids are defined according to the location of first double bond from their methyl end. Alpha-linolenic acid (ALA) is an example of n-3 fatty acid. It is denoted as 18:3n-3 which indicates that this fatty acid has three double bonds in 18 carbon chain length and the first double bond is located at third carbon from the methyl (omega) end. Linoleic acid (LA) is an example of n-6 fatty acids. It can be expressed by nomenclature as 18:2n-6 which indicates that this fatty acid has two double bonds in the chain length of 18 carbon atoms, and the first double bond is at the sixth carbon position from the methyl (omega) end. n/ω-3, n/ω-6 and n/ω-9 are the most important families, in term of an extent of occurrence and human health and nutrition. All of these are metabolised using the same group of enzymes (Ruxton et al. 2004), (**Figure 1.4**).

LA (18:2 ω-6) is a major fatty acid, present in plant lipids. Animals get it mostly from the dietary plant oils. Throughout the animal kingdom, arachidonic acid (20:4 ω-6) is found as a major component of membrane phospholipids, but very less amount of it is present in the diet. ALA (18:3 ω-3) is present in higher plants (soya bean oil and rapeseed oils) and algae. Eicosapentaenoic (EPA; 20:5 ω-3) and docosahexaenoic acids (DHA; 22:6 ω-3) are chief fatty acids of marine algae, fish oils and fatty fish. DHA is found in higher concentrations, especially in the phospholipids in the brain, retina and testes. Besides their vital role as an integral part of the cell membranes, PUFAs serve as the major constituents of plasma lipoprotein phospholipids, triglycerides, and cholesterol esters. ω-6 and ω-3 fatty acids cannot be inter-convertible, and both are essential nutrients (Le et al. 2009).

CH_3 - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - $(CH_2)_5$ - CH_2 - COOH

α-linolenic acid (ALA, C18:3n-3)

CH_3 - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - $(CH_2)_2$ - CH_2 - COOH

Eicosapentaenoic acid (EPA, C20:5n-3)

CH_3 - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH_2 - COOH

Docosahexaenoic acid (DHA, C22:6n-3)

CH_3 - CH_2 - CH_2 - CH_2 - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - $(CH_2)_5$ - CH_2 - COOH

Linoleic acid (LA, C18:2n-6)

CH_3 - $(CH_2)_3$ - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - CH_2 - CH = CH - $(CH_2)_2$ - CH_2 - COOH

Arachidonic acid (AA, C20:4n-6)

Figure 1.4. Nutritionally important polyunsaturated fatty acids.

1.2.4 Dietary sources of LCPUFAs

The n-3 and n-6 PUFAs are essential fatty acids (cannot be synthesised in the body) derived mainly from food. Eicosapentaenoic (EPA), docosapentaenoic (DPA) and docosahexaenoic (DHA) acid are ω-3 LCPUFAs that are synthesised in the human body from ALA. Arachidonic acid (AA) is another physiologically significant n-6 LCPUFA that is derived from LA. Since LA is abundant in the human diet, the quantity of AA available almost always exceeds the level required to maintain the metabolic needs. ω-6 fatty acids are abundantly present in liquid vegetable oils, including soybean, corn, safflower oil and cottonseed oils (Calder 2015). In rodents, AA alone has been reported to be as effective as LA in preventing symptoms of essential fatty acids deficiency (EFAD), (Thomasson, 1962; Hansen et a1. 1986). These studies demonstrate that AA can replace LA as the sole source of dietary omega-6 fatty acids (Le et a1. 2009). Unlike n-6 fatty acids, n-3 fatty acids do not occur in large amounts in plants food and western human diet. Moreover, ALA conversion process to EPA and DHA in humans is not efficient as it is only converted 5-10% to EPA and merely 2-5% to DHA (Arterburn et a1. 2006).

N-3 fatty acids are abundantly present in fish and shellfish. In fact, fish-oil supplements typically contain 30-50% of n-3 Fatty acids (Russo 2009). Therefore, major contributors of n-3 PUFAs in the diet are fish and other seafood. Some other dietary sources are chicken, eggs, soybean oil and canola oil. Other food products that provide fewer amounts of n-3 PUFAs include legumes, green vegetables, cauliflower, ground beef and whole milk (Burdge and Calder 2005). The main sources of n-3 LCPUFAs are fish and other aquatic animals. n-3 LUPUFAs produce by phytoplankton and other aquatic plants in the deep sea which are consumed by fish and other oceanic organisms regularly (Arterburn et al. 2006). Some fish and shellfish like salmon, herring, sardines and tuna have a significant quantity of EPA and DHA. Some researchers investigated that some fungi and alga are also good sources of DHA, and these have been cultivated, commercialised and mass-produced to supply the infant formula and maternal health industry (Harris 2004; Howe et al. 2006).

1.2.5 Consumption of n-3 fatty acids and recommendations

As per WHO suggestions, the dietary recommendation of n-3 PUFAs for the general population is 0.3 to 0.5 gm/day of EPA and DHA, and 0.8 to 1.1gm/day of ALA (WHO 2008). During pregnancy, DHA intake should be increased to 0.2 gm/day from seafood or some other sources of omega-3 LCPUFAs (Koletzko et al. 2007). The current adequate intake (AI) for ALA issued by the Institute of Medicine of the National Academies, USA (2005) is 1.6gm/day for men 19-47 years and 1.1gm/day for women 19-47 years. The physiological potency of DHA and EPA is greater than ALA. Moreover, there is a substantive increase in the evidence about the health benefits of omega-3 LCPUFAs (Mozaffarian and Rimm 2006). The recommendations of International Society for the Study of Fatty Acids and Lipids (ISSFAL) for minimum DHA and EPA intake is 500 mg/day (ISSFAL 2004). In USA, the total intake of omega-3 fatty acids is 1.6 g/d approximately, and only 0.1-0.2 g/d is coming from EPA and DHA (Kris-Etherton et al. 2000). In Japan, n-3 PUFAs intake is about 1-2 g/d (Sugano & Hirahara 2000). In Canada, the recommendations on total n-3 fatty acid intake is 1.2-1.6 g/d (Scientific Review Committee 1990).

In the UK, the Committee on Medical Aspects of Food and Nutrition Policy recommends that the intake of eicosapentaenoic acid and docosahexaenoic acid be 0.2 g/d or 1.5 g/wk (Kris-Etherton et al. 2002). The ISSAFL has proposed an adequate intake of EPA plus DHA to be 0.65 mg per day and even more in the case of pregnant and lactating women (Kolanowski et al. 2007; Arab-Tehrany et al. 2012). Intake of polyunsaturated fat appears higher among South Asians with P:S (PUFA: SFA) ratios than the British population (McKeigue et al. 1985, 1988; Reddy and Sanders 1992; Smith et at. 1993; Sevak et al. 1994). In a study of Gujerati Hindus, McKeigue et al. (1985) found that LA accounted for most of the high polyunsaturated fat intakes, as would be expected from a mainly vegetarian population. In contrast, dietary intakes of omega-3 LCPUFAs and their levels in plasma lipids were low. However, levels of omega-3 long-chain fatty acids in Muslim Bangladeshis in East London were high, presumably reflecting a higher intake of fatty fish in the diet (McKeigue and Marmot 1988; 1991). Another important and controversial point of view regarding the n-6/n-3 fatty acids is their ration role in health and disease (Crawford 2000; Griffin 2008; Simopoulos 2009). Nevertheless, it can be stated that an adequate intake of both n-3 and n-6 fatty acids, is essential for good health (Willett 2007).

1.2.6 LCPUFAs and cell membrane

Cell membranes enclose the cell and separate cell contents from the extracellular environment. They do not only serve as barriers to protect the cells from the outside environment but also play important roles in cell shape maintenance, solute transport and signal transduction in and out of the cell, cell-cell identification and communication. These membranes consist of two layers of amphipathic lipids, proteins and carbohydrates. However, the proportions of these components vary wildly between cell types, as well as between the same cells of different animal species. In mammalian cells, the lipid bilayers are composed of phospholipids, sphingolipids, cholesterol, and glycolipids, among which cholesterol occupies 30%, glycolipids 5% while phospholipids (61%) are the most abundant. The lipid fatty acid configuration of the membranes influences their physical properties, particularly the membrane fluidity (i.e. packing of lipids in the membrane bilayer). Which is determined by; 1) the nature of fatty acid chains; 2) the amount of cholesterol and 3) interaction of both polar and non-polar lipids and between lipids and proteins **(**Simons and Sampaio 2011; van Meer and de Kroon 2011).

According to Lauritzen et al. (Lauritzen et al. 2001), LCPUFAs are acylated to membrane phospholipids often in sn-2 position and make-up about 21-36% of the total fatty acid in cell membranes. Various processes influence distribution of the LCPUFAs in plasma and red blood cells such as dietary intake, intestinal absorption, metabolism and storage, and exchange among compartments, however, the typical composition of each lipid class is distinctive. In human plasma, CPG (69% of PLs), cholesterol esters (28% of total cholesterol) and triglycerides are dominant lipids classes. Whereas, in RBC, phosphatidylcholine (CPG, 29%), sphingomyelin (26%), phosphatidylethanolamine (EPG, 31%); and SPG and phosphatidylinositol (Pl),(13.2 %) are the major classes of phospholipids (Rise et al. 2007).

1.2.6.1 Cell membrane structure and organisation

Garth Nicolson and Jonathan Singer suggested the fluid mosaic model of membrane structure in 1972. It is now broadly accepted as the basic model for the organisation of all biological membranes. In this model, membranes are observed as two-dimensional fluids in which proteins are embedded in lipid bilayers (Cooper 2000). This membrane model has proved to be a very useful hypothesis in explaining many phenomena taking place in biological membranes, notably the distribution of molecular components in the membrane (Vereb et al. 2003).

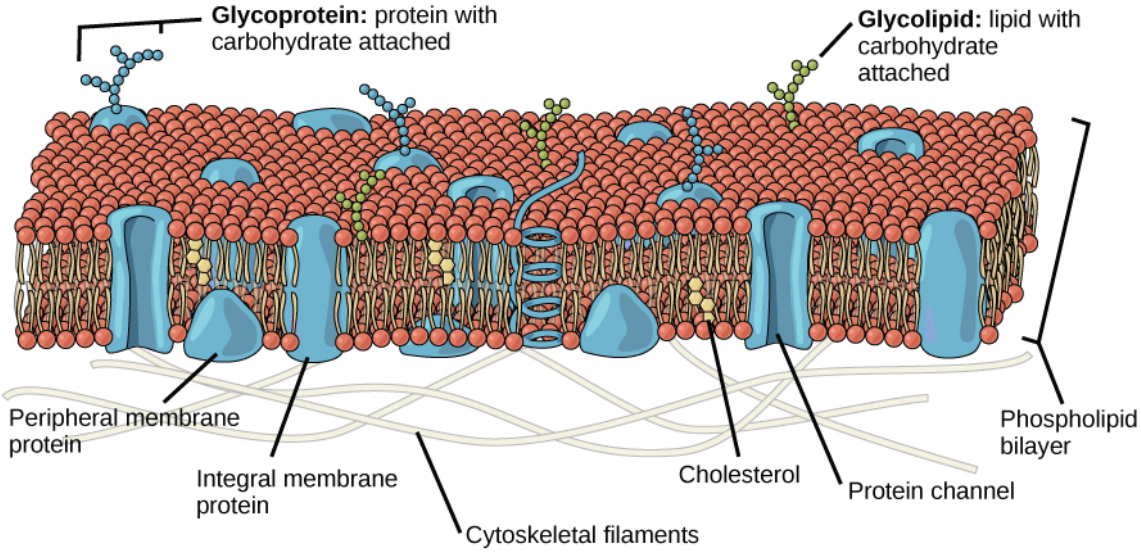

Figure 1.5. Schematic cross-sectional view of cell membrane (adopted from https://en.wikipedia.org/wiki/Fluid_mosaic_model).

The core structure of cell membranes establishes on the lipid bilayer which is the oldest but still approved molecular model of cellular structures (Gorter and Grendel 1925). In this model, the membrane is composed of two opposing lipid leaflets, with membrane proteins inserted across the bilayer or bound to either side of the leaflets

(**Figure 1.5**). It is, of course, a self-assembled system in itself. Membrane lipids have a polar hydrophilic phospholipid head group and hydrophobic tails (fatty acyl groups). This amphipathic character drives them to self-assemble into a bilayer, with the hydrophilic heads facing outside into the aqueous environment and the hydrophobic tails are hidden inside (pointed towards each other). This bilayer serves as a barrier for polar and charged solutes, helping to maintain electrochemical gradients across the membrane (van Meer and de Kroon 2011).

Plasma membranes of animal cells consist of four major species of phospholipids; choline phosphoglyceride (CPG/PC), ethanolamine phosphoglyceride (EPG/PE), Serine phosphoglyceride (SPG/PS), and sphingomyelin (SPM/SM). Almost half of the lipids in most membranes consist of these above-mentioned phospholipids, scattered asymmetrically between the two halves of the membrane lipid bilayer. Outer leaflet of the plasma membrane is mainly composed of choline-phospholipids (CPG and SPM) whereas amino-phospholipids (EPG and SPG) are the predominant phospholipids of the inner leaflet. A fifth phospholipid, phosphatidylinositol (PI), is also confined to the inner half of the membrane. Quantitatively, PI is a minor membrane component, but it plays a significant role in cell signalling. The head groups of both SPG and PI are negatively charged, so their dominance in the inner leaflet yields a net negative charge on the cytosolic surface of the plasma membrane (Cooper 2000).

1.2.6.2 Membrane lipids

It becomes increasingly evident that membrane lipids, independently and together with proteins, play a crucial role in the regulation of cell functions (Williamson et al. 2003; Lee 2005). It includes; cellular metabolism (Di Paolo and De Camilli 2006; Worgall 2008), signal transduction (Wymann and Schneiter 2008), regulation of gene expression (Sampath and Ntambi 2004; Sampath and Ntambi 2005) and are the major determinant of cell membrane fluidity and permeability (van Meer et al. 2008). The major structural lipids in mammals membranes are the glycerophospholipids, sphingolipids and sterols, particularly cholesterol, the main component of animal cell membrane lipids (van Meer et al. 2008; Mannock et al. 2010).

1.2.6.2.1 Membrane Sphingolipids

Sphingolipids are vital cell components, mainly residing in the external layer of the plasma membrane. Sphingosine is an 18-carbon monounsaturated alcohol containing an amino (-NH_2) group. A long-chain fatty acid (22-26 carbons) is attached to sphingosine (2-amino-1,3-dihydroxy-octadec-4-ene) to its -NH_2 group (amide linkage) forming what is called ceramide (**Figure 1.6**). Thus, ceramide is sphingosine-fatty acid complex (Merrill 2002). The major sphingolipids in mammalian cells are sphingomyelins (SMs) and the glycosphingolipids (GSLs), (Sonnino et al. 2006).

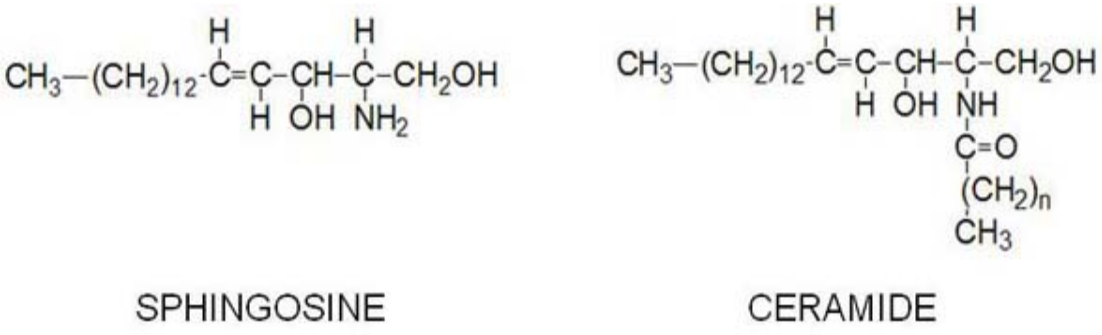

Figure 1.6. The structure of sphingosine and ceramide (Narayan and Thomas 2011).

1.2.6.2.1.1 Sphingomyelin

Sphingomyelins (SPM/SMs) constitute the most common sphingolipids in mammalian membranes (Talbott et al. 2000). These molecules have the structure ceramide-phosphocholine (polar head group) or ceramide-phosphoethanolamine (polar head group). The acyl group is generally saturated (16:0, 18:0, 22:0, 24:0) or monosaturated (18:1, 20:1, 22:1, 24:1) fatty acids (Byrdwell and Perry 2007). Sphingomyelin (ceramide-1- phosphorylcholine) is most abundantly present in the

outer layer of the plasma membrane (Ramstedt and Slotte 2002; Daleke 2008). Tissues in which SPM is the predominant phospholipid include the sheath of nerve cell axons, myelin (25%) and erythrocytes (18%), (Ramstedt and Slotte 2002). SPM synthesis occurs in the lumen of the Golgi as well as on the cell membrane (Huitema et al. 2004). The synthesis process which is catalysed by SM synthase (SMS) involves the transfer of phosphocholine from phosphatidylcholine onto ceramide, yielding diacylglycerol in the process (Villani et al. 2008; Gault et al. 2010). Because SM Synthase activity directly regulates the level of ceramide and diacylglycerol, it has also been suggested to play a vital role in regulating cellular functions (Gault et al. 2010). Sphingomyelin's high packing density and affinity for sterols helps in providing a rigid barrier to the extracellular environment and plays a role in the formation of lipid rafts, signal transduction and membrane trafficking (Holthuis et al. 200l; Ohanian and Ohanian 2001; Tafesse et al. 2007), (**Figure 1.7**).

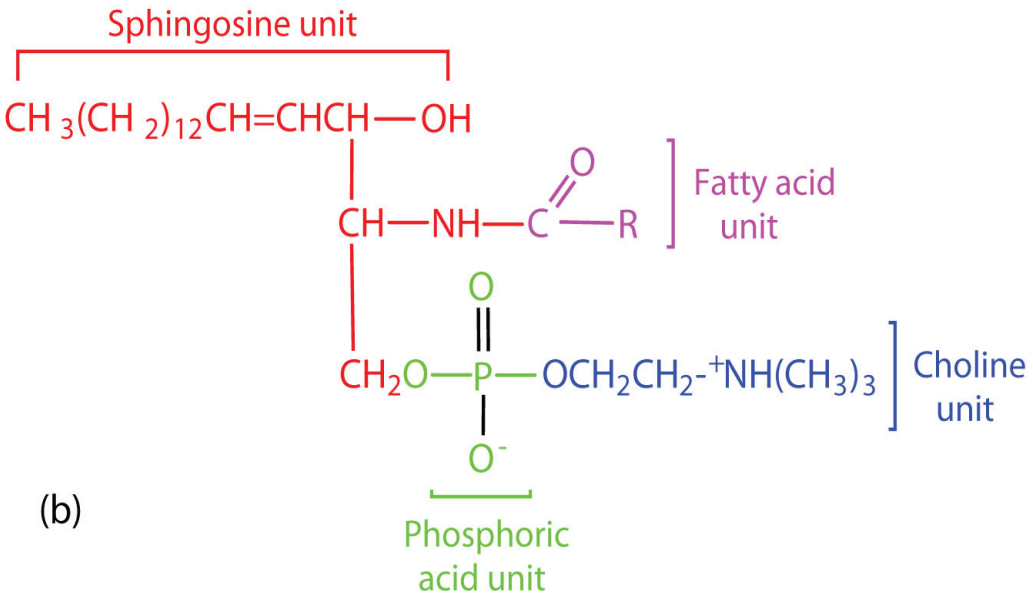

Figure 1.7. Structure of sphingomyelin (adopted from http://2012books.lardbucket.org/books/introduction-to-chemistry-general-organic-and-biological/s20-03-membranes-and-membrane-lipids.html).

1.2.6.2.1.2 Glycosphingolipids

Glycosphingolipids (GSLs) contain mono-, di- or oligosaccharides, based on glucosylceramide (GlcCer) and sometimes galactosylceramide (GalCer), (van Meer and Lisman 2002). In other words, GSLs are formed when one or more sugars are attached to ceramide at its terminal -OH group (Sonnino et al. 2006). The major GSLs in animal cell membranes are cerebrosides and gangliosides (Hakomori 2003). Cerebrosides also called monoglycosylceramides have the ceramide linked through an amide bond to glucose (glucocerebroside) or galactose (galactocerebrosides). Cerebrosides particularly occur in neuronal tissues (Tan and Chen 2003), and they appear to regulate the specific aspects of neuronal differentiation, proliferation, survival and apoptosis (Buccoliero and Futerman 2003). Glycosphingolipids are involved in the regulation of signalling through the growth factor and adhesion receptors (Odintsova et al. 2006; Sonnino and Prinetti 2010).

1.2.6.2.2 Cholesterol

Cholesterol is the most abundant animal sterol. It is a major and essential lipid component of the plasma membranes of the cells of higher animals. It is also found in lower concentrations in certain intracellular membranes in vesicular communication with the plasma membrane (Mannock et al. 2010). It typically accounts for 20-25% of the lipid molecules in the plasma membrane (Ikonen 2008), but it can increase up to 50% in red blood cells (Rog et aI. 2009). Structurally, cholesterol is a steroid built from four linked hydrocarbon rings attached to hydrocarbon tail at one end, and the hydroxyl group to the other end (**Figure 1.8**). Cholesterol affects cellular processes by interacting both with other membrane lipids and with specific proteins. Due to the rigid sterol backbone, cholesterol is preferably positioned in close proximity to the saturated hydrocarbon chains of neighbouring lipids. Therefore, resulting in increased lateral ordering of membrane lipids, and consequently affects the biophysical properties of the membrane, by decreasing fluidity and reducing the permeability of polar molecules (Simons and Vaz 2004; Ikonen 2008). Moreover, sterol-induced alterations in membrane biophysical properties and direct interaction with proteins could affect the cell membrane protein functions and cell signalling (Lee 2004; Olkkonen et al. 2006; Raychaudhuri and Prinz 2010).

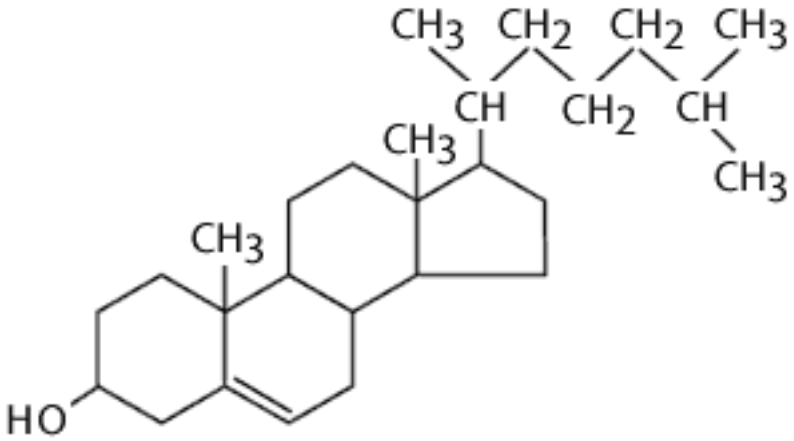

Figure 1.8. Structure of cholesterol (Chiang et al. 2007).

1.2.6.2.3 Phosphoglycerides

Phosphoglycerides are the most abundant cell membrane lipids in virtually all mammalian membranes. Phosphoglycerides consist of a glycerol core binding to a phosphate group (hydrophilic head) and two fatty acids (hydrophobic tail). According to the structure of the head group, phosphoglycerides are classified into choline (CPG), ethanolamine (EPG), serine (SPG), and inositol phosphoglycerides (IPG), phosphatidic acid (PA), phosphatidylglycerol (PG) and cardiolipin (**Figure 1.9**). Choline phosphoglyceride accounts for >50% of the phospholipids in most eukaryotic membranes (van Meer et al. 2008). In phosphoglycerides, the hydrocarbon chain at the sn2 position is always ester-linked to the glycerol moiety, while the sn1 position can be linked via an ester, ether or a vinyl ether bond. The length of the alkyl chain typically varies from 14 to 24 carbons and the number of double bonds from 0 to 6. The alkyl chain in the sn1 position is usually saturated or monounsaturated, while that

in the sn2 position is often polyunsaturated (Hermansson et al. 2011). Because of the large number of different alkyl chain combinations, each phospholipid class, in turn, consists of numerous molecular species that have the same head group but differ in respect of acyl chains (Han and Gross 2005; Taguchi and Ishikawa 2010). Besides serving as the key structural component of membranes, many phosphoglycerides also have other functions particularly in signalling transduction (O'keefe 2002; Fernandis and Wenk 2007; Meyer zu Heringdorf and Jakobs 2007; Guan and Wenk 2008). A brief description of various types of phosphoglycerides is given below;

- Phosphatidic acid (PA): In these acids, one of the fatty acids of a triacylglycerol is replaced by phosphoric acid. These are parent compounds of all glycerophospholipids (Vance and Vance 2004).
- Choline phosphoglycerides (CPG): These are derivatives of L-phosphatidic acid in which choline (a nitrogenous base) joins to phosphoric acid (phosphatidic acid + choline = CPG). In other words, these are phosphatidylcholines. Choline is trimethylethanolamine and is a quaternary ammonium compound which is as strongly basic as NaOH. Both of its structural fatty acids may be saturated/unsaturated, or one may be saturated while the other is unsaturated. They are the most abundant of the phospholipids in cell membranes, serum and bile.
- Ethanolamine phosphoglycerides (EPG): These are structurally identical with CPG except that the base choline is replaced by ethanolamine, forming phosphatidyl-ethanolamine.
- Serine phosphoglycerides (SPG): These are structurally identical with CPG except that the base choline is replaced by serine, forming phosphatidyl-serine.
- Inositol phosphoglycerides (IPG): Contain inositol base (Smith et al. 2005).

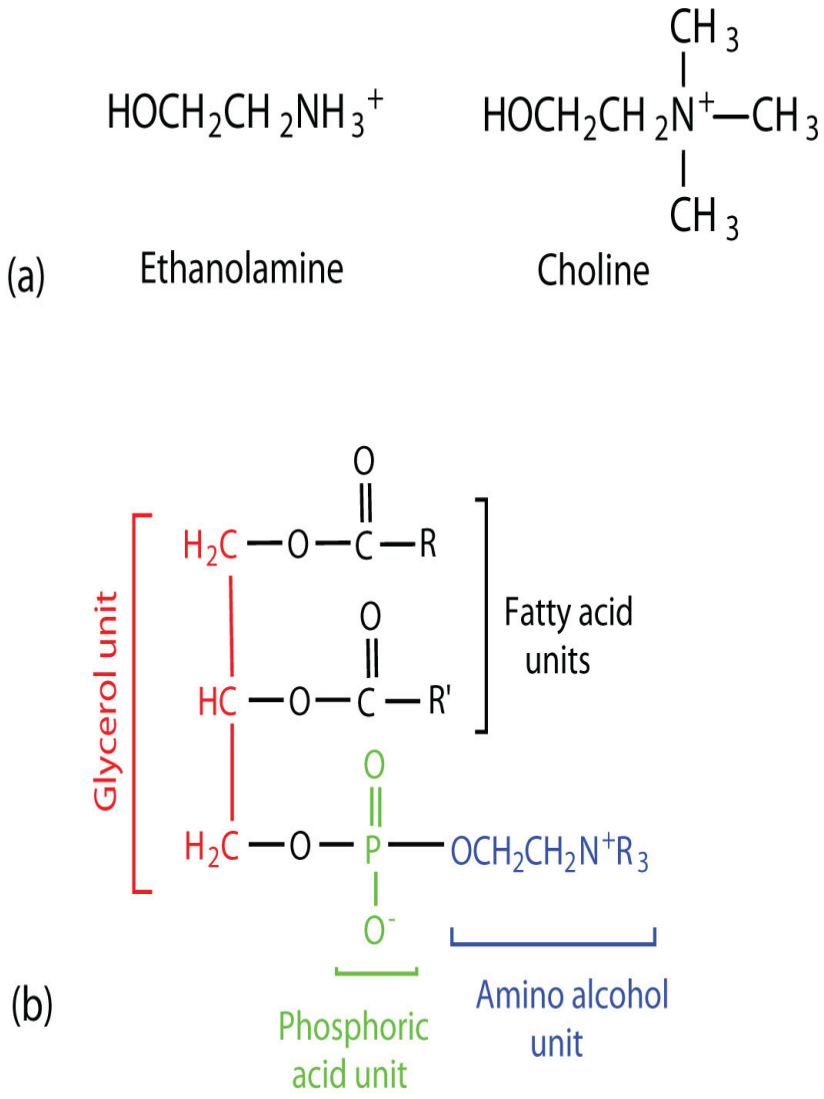

Figure 1.9. Choline and ethanolamine phosphoglycerides located in cell membranes (adopted from http://www.ncbi.nlm.nih.gov/books/NBK22361/).

1.2.7 Biosynthesis of long chain polyunsaturated fatty acids

When obtained from the diet, LA and ALA are further metabolized by Δ^6 desaturation (process of elimination of two hydrogen atoms from the fatty acid carbon chain), elongation (process of addition of two carbon atoms in the hydrocarbon chain of fatty acid), and Δ^5 desaturation to form arachidonic acid (AA) and eicosapentaenoic acid (EPA), respectively. In vivo, LA (18:2n-6) is desaturated by the action of Δ^6 desaturase to γ-linolenic acid (GLA, 18:3n-6) by inserting a double bond between the sixth and seventh carbon. GLA is then converted to dihomo-γ-linolenic acid (DHGLA, 20:3n-6) by the action of elongase enzyme (with an addition of two carbons). DHGLA is further transformed to AA (C20:4n-6) by Δ^5 desaturase, with the addition of a double bond between fifth and sixth carbons. Similar to the ω-6 pathway, the ω-3 pathway is mediated by the same set of enzymes that convert LA to AA. In the ω-3 pathway, ALA is transformed by Δ^6 desaturase, then elongated and desaturated to EPA (C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3), and docosahexaenoic acid (DHA, C22:6n-3), respectively (Ferdinandusse et al. 2001; Sprecher 2002; Le et al. 2009), (**Figure 1.10**).

The formation of LCPUFAs can take place in multiple organs e.g. brain (Moore et al. 1991), retina (Rotstein et al. 1996) and intestines (Garg et al. 1988) but mainly in liver (Voss et al. 2000). All reactions occur in the endoplasmic reticulum except the final reaction which takes place in the peroxisome, resulting in the formation of DHA and osbond acid (C22:5n-6) respectively. The formation of osbond acid and DHA was earlier thought to be catalysed by a Δ^4 desaturase, but is in fact, a retro-conversion: elongation and Δ^6 desaturation followed by translocation to the peroxisome and β-oxidation shortening the acyl chain to a 22 carbon LUPUFAs (Sprecher 2002).

If there is an insufficient supply of LCPUFAs to meet the physiological requirements, the body starts to synthesise certain other fatty acids with a similar molecular structure but without the same functions. These fatty acids are not present normally in the body (or present in low concentrations) and can, therefore, be used as LCPUFAs status markers. A general deficiency of LCPUFAs is indicated by a higher level of mead acid (C20:3 n-9). Deficiency of DHA results in increased production of osbond acid (C22:5 n-6), (Burdge and Calder 2005).

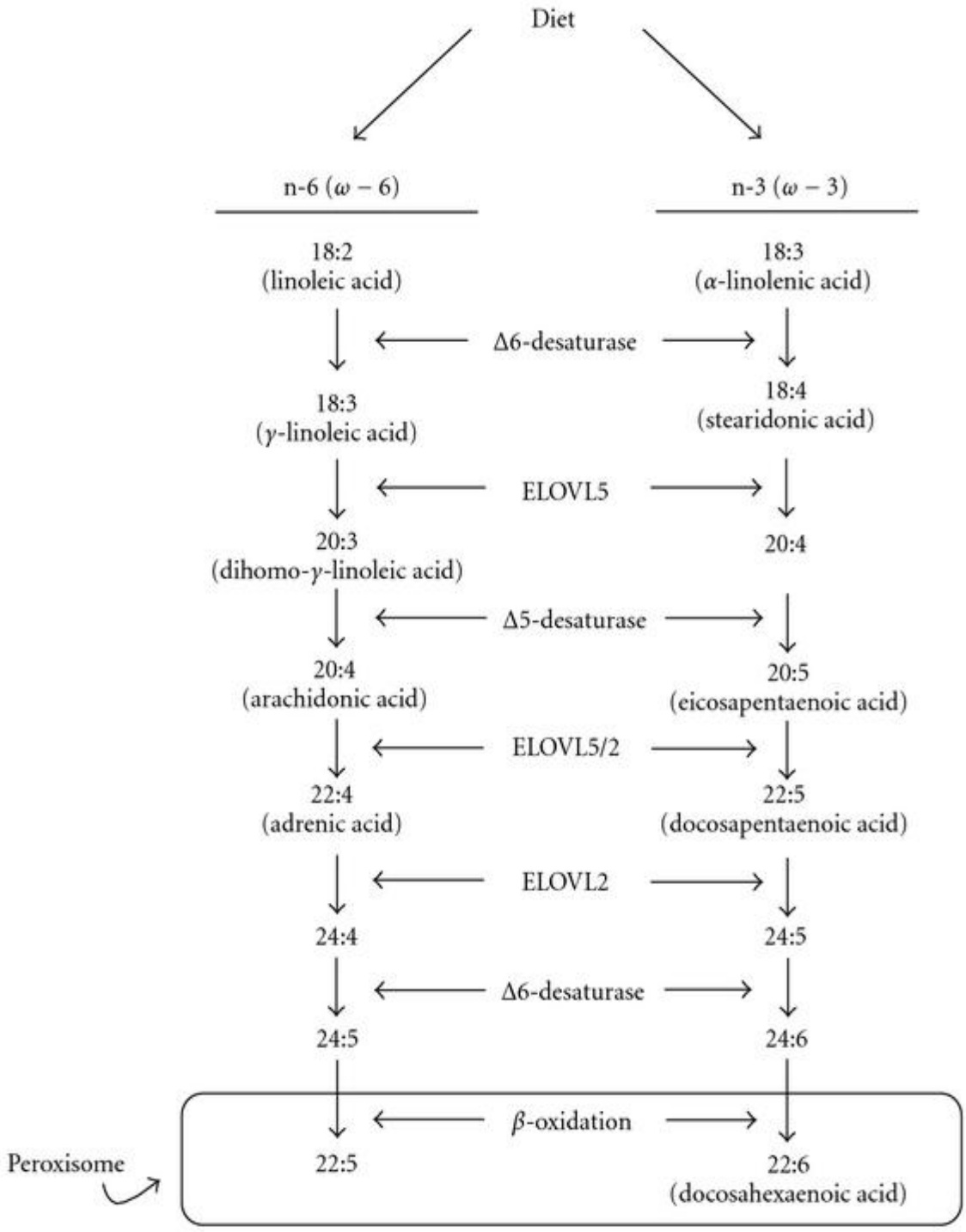

Figure 1.10. A general pathway for the conversion of LA and ALA into their LCPUFAs (Innis 2003).

1.2.8 Biomedical importance of ω/n-6 and ω/n-3 LCPUFAs

1.2.8.1 Biomedical importance of ω-6 (arachidonic acid)

The AA, ω-6 fatty acid, is present in all cell membranes and contributes up to 15% of the total fatty acids in phospholipids. Various studies have indicated that the low quantity of AA in maternal and cord blood is related to reduced head circumference and low birth weight (Crawford et al. 1989; Leaf et al. 1992), intra-uterine growth retardation (Vilbergsson et al. 1994) and impaired growth in preterm infants (Carlson et al. 1993). AA is a precursor of important signalling compounds called eicosanoids which include prostaglandins (PGs), prostacyclins, thromboxanes, leukotrienes and lipoxins (De Caterina and Basta 2001; Funk 2001; Calder et al. 2015[b]; Turcotte et al. 2015). These biologically active compounds have various roles in the regulation of immune response, inflammation and pain, smooth muscle contraction, platelet aggregation, renal electrolyte regulation and tumour cell proliferation (Vachier et al. 2002; Ferrucci et al. 2005; Simopoulos 2009; Turcotte et al. 2015).

1.2.8.2 Biomedical importance of ω-3 (EPA and DHA)

Cell membranes contain modest amounts of EPA and greater amounts of DHA. An exception is membranes of the brain (grey matter) and eye (rod outer segments), which contain high amounts of DHA (Crawford et al. 1976; Skinner et al. 1993; Makrides et al. 1994). Increased intake of ω-3 LCPUFAs (particularly EPA and DHA) is characterised by increased amounts of these fatty acids in membranes of many cell types (Calder 2014). Many but not all of the functional effects of ω-3 LCPUFAs rely on their incorporation into cell membrane phospholipids (Calder 2014). The structure of ω -3 LCPUFAs, particularly DHA, means that they have a strong influence on the physical properties of membranes into which they are incorporated, on membrane protein function, and on lipid raft formation (Calder 2015[a]). The vital role of DHA in visual function relates explicitly to the environment that DHA-rich phospholipids provide for rhodopsin, which optimises the function of this protein in signal transduction (Niu et al. 2004). As a result of their effects on membrane-generated intracellular signals, EPA and DHA can modulate transcription factor activation and, subsequently, gene expression patterns (Gottlicher et al. 1992; Novak et al. 2003; Clarke 2004; Deckelbaumet al. 2006; Jump 2008; Calder 2012). Effects of ω-3 LCPUFAs on transcription factor activation and gene expression are central to their

role in controlling inflammation, fatty acid and triacylglycerol metabolism, and adipocyte differentiation (Calder 2012; Calder 2014) Calder 2015[a]). The replacement of cell membrane AA by EPA and DHA influences the pattern of lipid mediators produced (Calder 2015[a]). As a result, EPA and DHA can influence inflammation, immune function, and blood clotting, vasoconstriction, and bone turnover, among other processes. EPA and DHA may also affect cell function by mechanisms that do not involve their incorporation into cell membranes. For example, non-esterified (NE) EPA and DHA can act directly via G protein-coupled receptors (GPRs) that exhibit some specificity for ω-3 LCPUFAs over other fatty acids as ligands. In particular, GPR120, which is highly expressed on inflammatory macrophages and adipocytes, was shown in cell culture experiments to play a central role in mediating the anti-inflammatory effects of DHA on macrophages and the insulin-sensitizing effects of DHA on adipocytes (Oh et al. 2010).

1.2.8.2.1 Effects on pregnancy and early childhood

In the context of pregnancy and child development, LCPUFAs, specifically DHA, has a vital role in neural, visual and behavioral development (Simopoulos 2002; Himmelfarb et al. 2007; Calder 2008). DHA is found in very high levels in the central nervous system and retina, especially in the grey matter and photoreceptors and believed to be essential for optimal development of these regions (Innis 2003; Arterburn et al. 2006; Bradbury 2011). DHA is preferentially transported to the infant during the 3rd trimester of gestation in humans and coincides with the later stages of the brain and retinal maturation (Carlson 2001; Innis 2005; Carlson 2009). It has been estimated that 67-75 mg/day of DHA is accumulated in-utero during the last trimester of gestation (Clandinin et al. 1980; Innis 2005; Henriksen et al. 2008). More recent studies in animals have suggested that early DHA exposure influences neural differentiation, neurotransmitter target finding and synaptogenesis during gestation (Innis and Owens 2001; McNamara and Carlson 2006). Several studies have evaluated the efficacy of maternal supplementation on infant's neurodevelopment (Lauritzen et al. 2004; Dunstan et al. 2007; Meldrum et al. 2012). It has been demonstrated that infant feeds which include DHA (and usually also AA) improve visual development in preterm infants and perhaps also improve visual and cognitive outcomes in term infants (Calder 2014). A recent meta-analysis has indicated an increase in mean gestational age and birth weight, and a decrease in the number of infants born before

37 weeks' gestation in mothers receiving n-3 LCPUFAs supplementation during pregnancy (Salvig and Lamont 2011).

LCPUFAs are the structural components of cell membranes. The retinal membrane phospholipids are comprised of over 47% DHA. Also, approximately 14% of brain DHA is present in grey matter (Diau et al. 2005; Heird and Lapillonne 2005). The availability of particular fatty acids during development is considered to be important in neurocognitive functions (Hadders-Algra et al. 2007; Bazan et al. 2011). High levels of LCPUFAs found in the basal ganglia, pre- and post- central cortices, hippocampus, and thalamus in neonatal baboons and rats suggesting that they affect sensorimotor integration and memory (Favreliere et al. 1998; Diau et al. 2005). It is now thought that EPA and DHA have important roles in the brain beyond infancy and may be important for brain function throughout the life course. Benefits of EPA and DHA have been shown in childhood attention, learning, or behavioral disorders (Richardson and Puri 2002; Stevens et al. 2003; Gustafsson et al. 2010; Milte et al. 2012; Perera et al. 2012; Yui et al. 2012) depression (Su et al. 2003), bipolar manic depression (Stoll et al. 1999), unipolar depressive disorder (Nemets et al. 2002), schizophrenia (Peet et al. 2001; Peet and Horrobin 2002) and aggression (Hamazaki et al. 2002).

1.2.8.2.2 Effects on blood lipid concentrations

Like other fatty acids, long chain ω-3 PUFAs affect blood lipid concentrations; EPA and DHA are particularly effective at lowering blood triacylglycerol concentrations. One important mechanism underlying this effect is reduced hepatic assembly and secretion of VLDLs, which are the main triacylglycerol- carrying lipoproteins in the fasting state. Hence, hepatic output of VLDL is decreased by EPA and DHA. There is also some evidence that EPA and DHA upregulate the expression of lipoprotein lipase in adipose tissue; an effect that would promote triacylglycerol clearance from the bloodstream. The triacylglycerol- lowering effect of EPA and DHA also probably involves decreased release of non-esterified fatty acids (NEFA) from adipose tissue, thereby reducing supply of substrate to the liver for triacylglycerol synthesis, and increased β-oxidation in skeletal and cardiac muscle thereby drawing circulating NEFAs away from the liver (Shearer et al. 2012). Acting through one or more, or perhaps all, of these mechanisms, EPA and DHA lower blood triacylglycerol

concentrations. EPA and DHA also influence the concentrations of cholesterol-carrying lipoproteins. They typically cause a small increase in both LDL and HDL cholesterol concentrations (Harris 1997; Woodman et al. 2002; Balk et al. 2006). EPA and DHA also increase LDL particle size, rendering LDL less atherogenic (Suzukawa et al. 1995; Woodman et al. 2003; Mori et al. 2000; Neff et al. 2011).

1.2.8.2.3 Antihypertensive effect

EPA and DHA affect many processes related to blood pressure, including the production of eicosanoids with vasoactive effects, the secretion of aldosterone, the generation of nitric oxide by the endothelium, vascular reactivity, and cardiac hemodynamics. Consequently, EPA and DHA lower blood pressure (both systolic and diastolic), as confirmed through several studies (Bao et al. 1998; Geleijnse et al. 2002; Balk et al. 2004; Hartweg et al. 2007; Miller et al. 2014). A study revealed that n-3 LCPUFAs consumption is related to the reduced risk of pre-eclampsia (Qui et al. 2006). The study conducted on patients with hypertension and diabetes revealed that increased level of DHA in blood led to the significant decrease in diastolic blood pressure and increased heart rate as compared to the control group which had low DHA level in their blood platelet membranes (Christensen et al. 2001).

1.2.8.2.4 Anti-inflammatory effect

EPA and DHA reduce inflammation. A key aspect of this anti-inflammatory action is the reduced production of eicosanoids from arachidonic acid and the increased production of proresolving mediators (resolvins and protectins) from EPA and DHA (Stulnig 2003; Calder 2015[a]). Many cohort studies report inverse associations between dietary intake or blood concentrations of EPA and DHA and the concentrations of a range of inflammatory markers, including cytokines, adhesion molecules, and acute phase proteins (Yli-Jama et al. 2002; Pischon et al. 2003; Ferrucci et al. 2006; Forouhi et al. 2014). Intervention studies with EPA and DHA have shown lowered concentrations of various cytokines, adhesion molecules, and acute phase proteins (Calder et al. 2011). EPA and DHA have also been shown to target inflammation in adipose tissue in humans (Itariu et al. 2012; Spencer et al. 2013). A placebo-controlled trial of n-3 supplementation in patients with exercise-induced bronchoconstriction had a significant improvement in symptoms and a reduction in the synthesis of inflammatory mediators (Mickleborough et al. 2003).

EPA and DHA are effective in some chronic inflammatory diseases such as rheumatoid arthritis (Volker et al. 2000; Cleland et al. 2003; James et al. 2003; Covington 2004; Miles and Calder 2012). Interestingly, other potential symptomatic benefits of n-3 fatty acids have also been observed in other inflammatory diseases, such as; osteoarthritis (Curtis et al. 2002), rheumatoid arthritis, inflammatory bowel diseases (Crohn and ulcerative colitis), (Belluzzi et al. 1996; Belluzzi 2002), some nephropathies (immunoglobulin A [IgA]), (Goumenos and Brown 2004) and infectious diseases such as hepatitis (Leu et al. 2004). EPA and DHA may play a role in reducing risk of childhood allergic diseases (Miles and Calder 2014). Related to their effects on inflammation, EPA and DHA can influence immune functions and defense against infections. However, effects of EPA and DHA on immune functions, apart from those related to inflammation, have not been consistently seen in humans. Nevertheless, some RCTs in children providing EPA + DHA, either added into milk (Thienprasert et al. 2009) or as supplements (Malan et al. 2015), have reported decreased illness resulting from respiratory infections.

1.2.8.2.5 Cardioprotective effect

Through effects on blood lipids and lipoproteins, vascular function, blood pressure and blood flow and inflammation, EPA and DHA lower risk of coronary vascular disease (CVD) and may also be used to treat existing CVD (Hu and Willett 2002; He et al. 2004; Von Schacky and Harris 2004). Substantial evidence from many studies has now accumulated, indicating that consumption of EPA and DHA reduces the risk of CVD outcomes in Western populations (London et al. 2007; Saravanan et al. 2010; De Caterina 2011). Chowdhury et al. (2014) reported a lowered risk of coronary outcomes in individuals consuming high compared with low amounts of EPA and DHA. Furthermore, individuals with high compared with low circulating concentrations of EPA, and DHA had lowered risk of coronary outcomes (Chowdhury et al. 2014). Thus, it seems likely that by establishing a better risk factor profile, EPA and DHA reduce the likelihood of atherosclerosis and so lower the risk of CVD. There has also been much interest in treating existing CVD with EPA and DHA. A number of studies published reported lower rates of death in patients with CVD who received supplemental EPA plus DHA, (Marchioli et al. 2002; Yokoyama et al. 2007; Gissi-HF et al. 2008). Three important mechanisms have been proposed to contribute to the therapeutic effect of EPA + DHA. The first is altered cardiac electrophysiology seen as

lower heart rate (Harris et al. 2008), increased heart rate variability (Xin et al. 2013), and fewer arrhythmias (Leaf and Xiao 2001). The second is an antithrombotic action resulting from the altered pattern of production of eicosanoid mediators that control platelet aggregation from AA and from EPA (von Schacky et al. 1985). The third mechanism is the well-documented anti-inflammatory effect of EPA (E-series resolvins, Rv) and DHA (D-series resolvins and neuroprotectin D1), which would serve to stabilize atherosclerotic plaques, preventing their rupture (Calder 2008; Serhan and Chiang 2008; Cawood et al. 2010).

1.2.8.2.6 Anticancerous effects

EPA and DHA exert a range of biological activities that may influence tumour cell proliferation and viability; for example, DHA can promote tumour cell apoptosis possibly through inducing oxidative stress (Gleissman et al. 2010; Merendino et al. 2013). EPA and DHA also reduce production of mediators such as prostaglandin E 2 that drive tumour cell proliferation and tumour growth (Wang and Dubois 2010; Vaughan et al. 2013). Anti-thrombotic effect of n-3 LCPUFAs was initially associated to EPA because of its competition with AA in cyclooxygenase (COX) and lipoxygenase (LOX) pathways. n-3 LCPUFAs (particularly EPA) obstruct the cyclooxygenase activity and its inhibition leads to the low level of prostaglandins and activates the lipoxygenase enzymes. The increased activity of lipoxygenases results in increased formation of hydroxyeicosatrienoic acids (HETE) and leukotriene B4 (LTB4), which are responsible for reducing the intensity of inflammation and pain and slow down the process of cancerous cells surpassing a tissue (Nomura et al. 2003). Through these effects, EPA and DHA can directly influence cancer cells and the tumour environment, and they can influence the host response to tumour bearing. Anti-inflammatory actions of EPA and DHA may also be important in preventing or slowing some steps in tumour initiation, particularly in some cancers such as colorectal cancer. Recent reviews provide in-depth analysis of the mechanisms by which EPA and DHA affect tumour cell proliferation, invasion, and metastasis (Gleissman et al. 2010; Merendino et al. 2013) and the ability of EPA and DHA to enhance the effectiveness of anticancer treatments (Merendino et al. 2013; Vaughan et al. 2013). Both DHA and EPA help in providing protection against tumor growths But, some human and animal studies conclude that DHA is the more effective anti-aggregatory agent than EPA in preventing transcription factor activator protein 1 (AP-1), which is

associated with the progress of cancerous growth (Liu et al. 2001; Woodman et al. 2003; Cottin et al. 2011)

1.2.8.2.7 Effects on insulin resistance and diabetes

Insulin resistance plays an important role in various chronic diseases including metabolic syndrome and type II diabetes. A growing body of evidence suggested that there is an inverse association between n-3 LCPUFAs and insulin resistance (IR). Anti-diabetic effects of PUFAs have been observed, including increased basal metabolic rate and fat oxidation (Jones and Schoeller 1988; Couet et al. 1997); however, some of these findings were resulted from studies comparing polyunsaturated: saturated fatty acid intake. Studies involving fish oil effects on the composition of human body and IR vary depending on the health of the subjects and the type of the study. Consequently, it has been hard to determine the effects of EPA and DHA on diabetes-related parameters during human trials (Anderson and David 2009). Animal studies involving EPA, DHA and IR likely to be more consistent and support an anti-diabetic effect. Numerous rodent studies revealed that EPA improves IR in numerous models of obesity and diabetes (Mori et al. 1997; Mori et al. 1999; Nobukata et al. 2000) and elevates systemic concentrations of insulin-sensitizing adiponectin (Flachs et al. 2006). An improved response to a glucose load was also reported in mice fed high-fat diets enriched in EPA and DHA (Ikemoto et al. 1996). Several studies evaluated fish oil feeding in sucrose-fed rats and noticed reduced peripheral IR, hyperglycemia and fat pad mass (Soria et al. 2002; Pighin et al. 2003) as well as increased insulin-stimulated glucose transport in supplemented animals (Peyron-Caso et al. 2002). EPA and DHA also observed to prevent alloxan-induced diabetes and restored the antioxidant status of various tissues to normal range in rats (Suresh and Das 2003). Both have shown to be more efficient than ALA at lowering plasma glucose plus insulin levels and improving insulin sensitivity (Andersen et al. 2008).

n-3 LCPUFAs are proposed to decrease the risk of insulin resistance by multiple means, few of which seem to be distinctively affected by n-3 fatty acids. EPA and DHA are favorably incorporated into cell membranes, hence increasing membrane fluidity. Which, in order, has been shown to raise the number of insulin receptors at the cell membrane and their affinity to insulin (Das 2005). Up-regulation of insulin

receptors decreases the insulin resistance and favorably modifies an individual's glycemic response, an effect that could potentially prevent or delay the onset of type II diabetes. Transcription factors are also involved in IR. Nuclear factor-kB (NF-kB) activation of endothelial cells was exhibited in response to hyperglycemia, though, EPA and DHA were exhibited to down-regulate NF- kB (Morigi et al. 1998). This could potentially mediate several vascular complications that result from chronically raised glucose levels seen in diabetics. Additionally, peroxisome proliferator-activated receptor gamma (PPARγ) is linked with the etiology of IR, as it raised the expression and translocation of glucose transporters (GLUT-1 and GLUT-4), thus, facilitating glucose uptake in adipocytes and the muscle cells (Kramer et al. 2001). EPA and DHA appear as ligands for PPARs and so, may have an anti-diabetic role. Furthermore, stimulation of PPARγ inhibits the expression of IR-promoting cytokines and simultaneously triggering an increase in the plasma concentrations of adiponectin (Gross and Staels 2007). This resulted in decreased blood glucose levels by improving insulin sensitivity and reducing liver glucose production (Yamauchi et al. 2001).

1.3 Diabetes mellitus

Diabetes mellitus (simply diabetes, DM) is characterised by different metabolic disorders in which an individual has high blood glucose levels, either because of the pancreas that does not produce sufficient insulin or due to the cells, which do not respond to the insulin produced. As a result, typical manifestations of diabetes; polyuria (frequent micturition), polydipsia (increased thirst) and polyphagia (increased hunger) are produced (Shoback 2011). Diabetes has different types, however, the long-term negative effects of chronic hyperglycemia on various organs; for example, kidneys (nephropathy), blood vessels (angiopathy), eyes (retinopathy), nerves (neuropathy) and heart remain similar (ADA 2012).

1.3.1 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance that begins or is first recognised during pregnancy and generally resolving shortly after delivery. This definition acknowledges the prospect that patients may have undiagnosed diabetes mellitus previously or might have developed diabetes as a coincidence with pregnancy. (ACOG 2001; ADA 2004; Diabetes Care 2008; NICE 2015). A woman is said to have GDM when the glucose intolerance continues beyond 24-28 weeks of gestation. GDM, depending upon the populace studied, influences 1-14% of all pregnant females (ADA 2012). GDM is frequently more common in populaces with a high occurrence of type II diabetes, for example, India and China (Hunt and Schuller 2007). It is acknowledged that GDM women have a significant risk of advancing to type II in later life (Kim et al. 2002).

Although, it is an eminent cause of pregnancy complications, but its epidemiology has not been studied systematically. An important problem is the distinction of GDM, as now defined, from pre-existing but un-diagnosed diabetes, so that the degree of clinical surveillance may have a major impact on the estimated prevalence of GDM in a given population. This is especially true in high-risk populations in which the onset of type II diabetes occurs at an early age. Furthermore, investigators use different screening programs and diagnostic criteria for GDM, making comparisons among studies difficult (King 1998; ACOG 2001; Hunt and Schuller 2007). Almost 700,000 women give birth in the UK each year, and up to 5% of them have either pre-existing diabetes or GDM. Of females who have diabetes

during pregnancy, it is estimated that around 87.5% have GDM (that may or may not resolve after pregnancy), 7.5% have type I diabetes, and the remaining 5% have type II diabetes. The prevalence of type I diabetes, and especially type II diabetes, has increased in recent years. The incidence of GDM is also increasing as a result of the high prevalence of obesity in the general population as well as more pregnancies in older women (NICE 2015).

As compared to Caucasians the frequency of GDM is two and four times greater in females of African and Asian origin, respectively (Toms et al. 1992; Koukkou et al. 1995). The impact of ethnic origin on the occurrence of GDM was assessed during a study in the UK, where the females were screened for GDM. Females from ethnic origins other than White had a higher incidence of GDM than White ones (2.9% vs. 0.4%). The relative risk of GDM in other ethnic groups compared to white females was: South East Asian 7.6, Black 3.1, Indian 11.3 and miscellaneous 5.9 (Dornhorst 1992).

1.3.1.1 Risk factors for GDM

Various maternal risk factors for the development of GDM have been described. The traditional and most often reported risk factors for GDM are increased maternal age, weight, and high parity, previous delivery of a macrosomic infant and a family history of diabetes (**Table 1.1**). These and other reported risk factors are described below.

1.3.1.1.1 Maternal risk factors

1.3.1.1.1.1 Older age has consistently been reported as a risk factor for GDM (Egeland et al. 2000; Jimenez-Moleon et al. 2000; Jolly et al. 2000; Bo et al. 2001; Lao et al. 2001; Xiong et al. 2001; Ben-Haroush et al. 2004; Kieffer et al. 2006; Silva et al. 2006; Berg et al. 2007).

1.3.1.1.1.2 Ethnicity has an influence on the occurrence of GDM. It differs considerably among ethnic groups in the USA: 9.7% in Asians, 7% in Latinas, 4.3% in African-Americans and 4.1% in Caucasians (Esakoff et al. 2005). Asian females residing in the USA have the high occurrence of GDM. Among them, the increased risk of GDM has been identified in females from Bangladesh with a risk ratio of 7.1 in comparison with non-Hispanic whites (Savitz et al. 2008). A study carried out in

London revealed that pregnant females with GDM from Bengali-Asians origin residing in an east London health district were more aged and of higher parity than the Caucasians and required insulin therapy more frequently. Persistence of abnormal glucose tolerance was shown by 20% of Bengali people as compared to the Caucasian group who presented no abnormalities (Toms et al. 1992).

1.3.1.1.1.3 Higher parity has also been stated as maternal risk factors for GDM. Grand multiparas (i.e. females with ≥ 5 deliveries) had more frequently an insulin-dependent GDM as compared to multiparas with 2-4 deliveries (Roman et al. 2004). The risk of GDM in grand multiparas was two-folds higher in comparison to females with 2-3 deliveries (Nassar et al. 2006).

1.3.1.1.1.4 Pre-pregnancy body weight is another reported risk factor of GDM. Well-known risk factors of GDM include overweight and obesity. Body mass index (BMI) of 25.0-29.9 kg/m^2 doubles the risk of GDM, and it becomes 6-folds higher at BMI ≥ 30 kg/m^2 when compared to women having normal BMI (Cnattingius and Lambe 2002). According to another study, obese pregnant women have more than doubled the risk of developing GDM, and it increases to four-folds with BMI ≥ 35 kg/m^2 as compared to women with BMI less than 30 kg/m^2 (Weiss et al. 2004). Another study has also identified the high BMI as a GDM risk factor (Kousta et al. 2000[b]).

1.3.1.1.1.5 Polycystic ovarian syndrome (PCOS) is more prevalent in GDM women (Kousta et al. 2000[b]; Koivunen et al. 2001; Mikola et al. 2001; Haver et al. 2003).

1.3.1.1.1.6 Short stature is another maternal risk factor described in some studies (Bo et al. 2001). The incidence of GDM was higher in Korean women shorter than 157cm (Jang et al. 1998). In Brazil, women shorter than 151cm demonstrated a 60% increase in GDM than women of 160cm height or above (Branchtein et al. 2000). Additionally, in Australia, GDM women were considerably shorter and have a lower leg-to-height percentage (Moses and Mackay 2004). South Asian and European women with previous GDM were shorter than control women from the same ethnic groups (Kousta et al. 2000[a]).

1.3.1.1.2 Genetic risk factors

There are at least ten genes where certain polymorphisms are linked with a high risk of GDM, most significantly TCF7L2 (Zhang et al. 2013).

Table 1.1. Maternal determinants, generally referred as" traditional risk factors for GDM (adopted from Ben-Haroush et al. 2004).

Risk Factors for the development of GDM	
1.	Prior GDM
2.	Pre-pregnancy obesity
3.	High risk ethnic group- South Asian, Aborginal, Hispanic
4.	Prior macrosomic infant (Birth weight > 4.5 kg)
5.	Maternal age > 35 years
6.	Polycystic ovarian syndrome
7.	Family history of T2DM in a first degree relative
8.	Multiple gestation
9.	Previous history of impaired fasting glucose or impaired glucose tolerance

1.3.1.1.3 Family history

Family history of diabetes mellitus is considered as another risk factor for GDM (Jimenez-Moleon et al. 2000; Ben-Haroush et al. 2004; Cypryk 2008). The first-degree heredity of type II DM was more prevalent in previous GDM than the control group (Holte et al. 1998). A family history of maternal type II diabetes mellitus is considerably more common among GDM women than a paternal family history (Egeland et al. 2000). Also, type II diabetes was significantly observed more in the

maternal-grand maternal line among GDM women compared to the paternal-grand paternal line (Harder et al. 2001).

1.3.1.1.4 Previous obstetric outcomes

Certain outcomes of previous pregnancies are also observed as risk factors for GDM in the consecutive pregnancies, for example, giving birth to a baby with macrosomia and previous GDM (Jimenez-Moleon et al. 2000). Though, previous macrosomia as a risk element for GDM has been argued recently as macrosomia may have some other causes, for example, obesity, multiparity, unnecessary weight gain in pregnancy and hereditary factors (Grassi and Giuliano 2000; Magenheim et al. 2007). However, it has been shown that pregnant women with the history of still birth and C-section have a high incidence of GDM (Xiong et al. 2001). Some studies show that the GDM women are more likely to have the recurrence of the disease in their subsequent pregnancies (Major et al. 1998; Spong et al. 1998; MacNeill et al. 2001).

1.3.1.1.5 Pregnancy factors

Some other risk factors for GDM have been recognised as raised blood pressure during pregnancy (Ma and Lao 2001), multiple pregnancies (Wein et al. 1992; Schwartz et al. 1999; Sivan et al. 2002) and elevated iron stores (Lao et al. 2001). Accelerated foetal development (Langer et al. 1991; Nordin et al. 2006) and polyhydramnios (Nobile de Santis et al. 2004; Nordin et al. 2006) may also raise the risk of GDM.

Added risk factors for GDM which are modifiable, include less physical activity, increased intake of dietary fat and life style habits (e.g. smoking and some drugs use), all badly influence insulin resistance. Wang et al. studied the aspect of increased body weight and polyunsaturated fat intake (Wang et al. 2000). They determined that both had an independent relationship with concentrations of glucose, such as increased body weight and reduced intake of polyunsaturated fat, both were predictive of the incidence of GDM.

1.3.1.2 Pathogenesis of gestational diabetes

The incidence of GDM has doubled over the last few years and is paralleling the obesity epidemic. GDM causes long-term implications for the subsequent development of type II diabetes in the mother and high risk for obesity and glucose intolerance in the children. As pregnancy advances, the increasing tissue resistance to insulin creates a demand for more insulin, and, if resistance becomes dominant due to impaired insulin secretion, hyperglycaemia develops. In the majority of such cases, it develops in the latter half of gestation, with insulin resistance progressively increases until delivery, when, in most cases, it disappears rapidly (Barbour et al. 2007).

It has been convincingly established that GDM arises as a result of a combination of insulin resistance and reduced insulin secretion. As per the definition of GDM, there are two important points for the discussion. First, pregnancy is normally attended by progressive insulin resistance that begins near mid-pregnancy and progresses through the third trimester till delivery. The insulin resistance develops from a combination of increased maternal adiposity and insulin-desensitizing effects of hormonal products of the placenta. The fact that insulin resistance rapidly decreases following delivery suggests that one of the contributors to this state of resistance are placental hormones. The second point is that pancreatic beta cells normally increase their insulin secretion to compensate for the insulin resistance of pregnancy. Consequently, the changes in blood glucose levels over the period of pregnancy are quite small as compared to the large changes in insulin sensitivity (Moshe Hod et al. 2008), (**Figure 1.11**).

1.3.1.2.1 Insulin resistance

The development of resistance to the glucose-lowering effects of insulin is a normal phenomenon of pregnancy. This physiological insulin resistance also appears in women with GDM. Though, it occurs on a background of chronic insulin resistance and to that the insulin resistance of pregnancy is partially additive. Thus, GDM pregnant women tend to have even greater insulin resistance than healthy pregnant women. In addition to that, insulin secretion is inadequate to compensate for the insulin resistance, leading to hyperglycaemia that is detected by routine glucose screening in pregnancy. Thus, chronic insulin resistance is a central component of the pathophysiology of GDM (Barbour et al. 2007).

Human pregnancy is characterised by a sequence of metabolic changes that enhance adipose tissue accretion in early pregnancy, followed by insulin resistance and facilitated lipolysis in later pregnancy. At the beginning of pregnancy, the insulin secretion increases, while insulin sensitivity is unchanged, decreased, or may even increase (Catalano et al. 1993; Catalano et al. 1999). However, in late pregnancy, maternal adipose tissue depots decline, while postprandial FFAs levels increase and insulin-mediated glucose disposal worsens by 40-60% as compared to pre-pregnancy (Catalano et al. 1999). The ability of insulin to suppress whole-body lipolysis is also reduced during late gestation (Homko et al. 1999). This insulin ability is further reduced in GDM women (Catalano et al. 2002), contributing to greater postprandial increases in FFA levels, increased hepatic glucose production, and high insulin resistance (Metzger et al. 1993; Buchanan et al. 1999; Catalano et al. 1999; Friedman et al. 1999). Catalano et al. (1991) determined an approximate decrease of 21% in insulin sensitivity appearing to 12-14 weeks of gestation and 56% decrease in insulin sensitivity appearing to 34-36 weeks. Others have shown similar results (Xiang et al. 1999; Buchanan et al. 2000; Buchanan et al. 2001).

Although many placental hormones have been suggested to reorganise maternal physiology to meet the foetal needs, the cellular mechanisms behind this complex transition remain unclear (Handwerger and Freemark 2000). Much effort has been invested in identifying the tissues that contribute to the insulin resistance of pregnancy. Findings in animal models indicate a 40% reduction in insulin-mediated glucose utilisation by skeletal muscle and a similar effect in cardiac muscle and fat cells (Leturque et al. 1986; Hauguel et al. 1988).

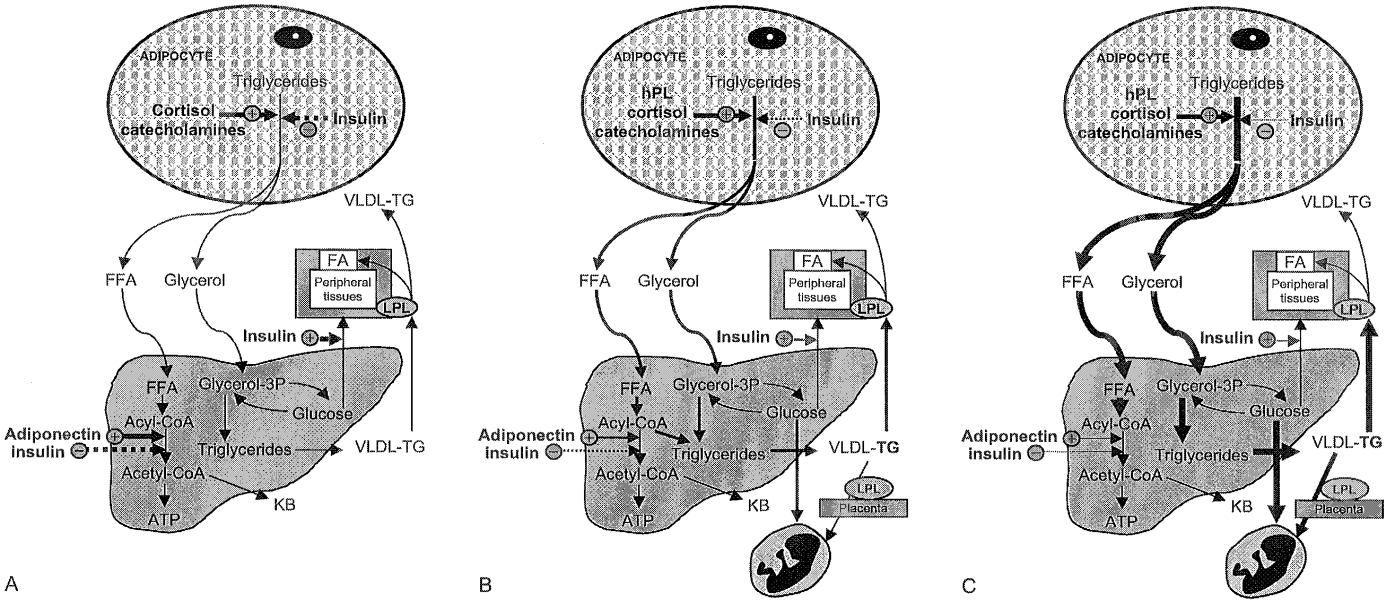

Figure 6.1 Intermediary metabolism in non-pregnant women (panel A), normal pregnancies (panel B) and pregnancies with GDM (panel C). The thickness of the lines relates the degree of stimulation (solid lines) or inhibition (dash lines) of either the metabolic pathway or the hormonal effects. TG: triglycerides; KB: ketone bodies; FFA: free fatty acids; FA: fatty acids; hPL: placental lactogen; LPL: lipoprotein lipase.

Figure 1.11. Intermediary metabolism in pregnancies complicated by gestational diabetes (Moshe Hod et al. 2008).

1.3.1.2.2 Hormonal effects

Pregnancy is accompanied by profound hormonal changes, which have a direct effect on carbohydrate tolerance. Reproductive hormones tend to increase during pregnancy, most of them contribute to insulin resistance and altered beta-cell function. In early gestation, both progesterone and oestrogen rise but their effects on insulin activity are counterbalanced. Progesterone causes insulin resistance whereas oestrogen is protective (Ryan and Ennes 1988; Gonzalez et al. 2000). Cortisol levels increase as pregnancy progresses and by the end of pregnancy concentrations are three-folds higher than in the non-pregnant state (Gibson and Tulchinski 1980). High levels of Cortisol are linked with increased hepatic glucose production, decreased phosphorylation of the insulin-receptor and profound insulin resistance (Rizza et al. 1982). During pregnancy, maternal prolactin levels increase 7-10 folds. Although, raised prolactin levels are not of pathophysiological importance in the development of GDM (Skouby et al. 1986). The levels of human placental lactogen (hPL) rise at the beginning of the 2nd trimester, causing a decrease in phosphorylation of insulin receptor substrate (IRS)-1 and severe insulin resistance (Ryan and Ennes 1988).

Adiponectin is a key insulin-sensitizing hormone produced by the adipose tissue. It is considerably lower in women with the history of GDM and declines further with advancing pregnancy, suggesting its likely involvement in the transition to insulin resistance (Ranheim et al. 2004; William et al. 2004; Winzer et al. 2004; Heitritter et al. 2005; Thyfault et al. 2005; Ategbo et al. 2006). In adipose tissue, the lipogenic transcription factor and PPAR-δ decreases in obese pregnant women that could shift genes in metabolic pathways to support increased lipolysis, hence accelerating adipose tissue insulin resistance (Bruun et al. 2003; Fasshauer et al. 2003). This transition to insulin resistance adds on to greater postprandial increases in the circulating FFAs and increased hepatic glucose production, which results in greater fuel availability to the foetus of the GDM women. Thus, like a perfect storm, the placental hormones, less adiponectin secretion, and excess lipolysis collaborate to cause profound insulin resistance in muscle, liver, and adipose tissue in GDM women.

1.3.1.2.3 Pancreatic beta-cell function

Insulin is a hormone that controls blood glucose concentration. Fasting plasma insulin increases gradually during pregnancy, and by the 3rd trimester, levels are 2-folds higher than before pregnancy. Women with GDM have fasting insulin levels equal to or higher than those of women with healthy pregnancies, with the highest levels occurring in obese GDM women. During normal pregnancy, oral and intravenous glucose tolerance deteriorates only slightly, despite the reduction in insulin sensitivity (Buchanan et al. 1990). The mechanism accountable for increase insulin secretion during gestation is not well understood. A chief contributing factor is an increase in the beta cell mass, a combination of hyperplasia and hypertrophy (Van Assche et al. 1987). The increased beta cell mass can contribute to the increased fasting insulin concentration despite normal or lowered fasting glucose concentrations in late gestation, and the enhanced insulin response to glucose during gestation (2-3 folds above non-pregnant levels). In most women, pancreatic insulin secretion rises to meet this need (to overcome insulin resistance), but in those with underlying beta cell defects, hyperglycaemia ensues (Swinn et al. 1995). GDM tends to be milder in subjects with a normal beta cell response, and they are at relatively low risk for developing diabetes in later life (Kjos et al. 1995).

1.3.1.2.4 Genetics, immunology and gestational diabetes mellitus

Increased plasma levels of TNF-α have also been linked to insulin resistance. The mechanism involved is probably related to a decrease in the insulin receptors (Hotamisligil et al. 1994; Kirwan et al. 2002). Some GDM women manifest evidence for autoimmunity towards beta cells (insulin auto-antibodies and anti-islet cell antibodies); though, the prevalence of such autoimmunity has been stated to be extremely low (< 10%), (Catalano et al. 1990; Damm et al. 1994). The parallel frequencies of HLA-DR2, -DR3 and -DR4 antigens in well pregnant women and GDM women, and the low incidence of markers for autoimmune destruction of the beta cells in GDM may exclude the possibility that GDM has an autoimmune origin (Ober et al. 1989; Vambergue et al. 1997).

1.3.1.2.5 Insulin signalling system in normal pregnancy and GDM

When insulin binds to the insulin receptor (IR), the activation of the tyrosine kinase (TK) enzyme on the beta subunit leads to increased phosphorylation of the cellular substrates. Insulin receptor substrate-1 (IRS-1) is a cytosolic protein that binds to the phosphorylated intracellular substrates and transmits the insulin signal downstream. The dispersal of the IRS proteins tends to be tissue specific. IRS-2 is more abundant in the liver and pancreas, while both IRS-1 and IRS-2 are widely spread in skeletal muscle. Insulin triggers the activation and binding of the lipid kinase enzyme, phosphatidylinositol (PI)-3-kinase, and its binding to IRS-1. The formation of PI is crucial for insulin action on the glucose transport. In GDM women, the skeletal muscle contains lower levels of IRS-1 protein and considerably less insulin-stimulated IRS-1 tyrosine phosphorylation, whereas the levels of IRS-2 protein are increased. These findings propose that the insulin resistance of GDM can be exerted through a reduction in insulin resistance cascade at the level of IRS proteins. The higher IRS-2 level may be a compensation for the reduced IRS-1 level (Shao et al. 2000).

The latest research suggests that the post-receptor mechanisms that contribute to insulin resistance of pregnancy are multifactorial, but are exerted at the beta subunit of the IR and the level of IRS-1. The resistance to insulin-mediated glucose transport appears to be greater in skeletal muscle of GDM women than from pregnancy alone (Garvey et al. 1993). IR tyrosine phosphorylation has also been reported to be impaired in muscle from obese GDM women (Garvey et al. 1992). Besides, overexpression of membrane plasma cell differentiation factor-1 (PC-1) may play a role in developing insulin resistance by inhibiting the TK activity of the IR (Garvey et al. 1993). In GDM women, PC-1 levels were significantly higher in skeletal muscle compared to healthy pregnant women (Goldfine et al. 1998; Shao et al. 2000).

GDM is a predictor of DM (mainly type II) later in life. Also, GDM is a predictor or even an early manifestation of the metabolic syndrome (insulin resistance). GDM is a risk factor for cardiovascular diseases and affected women should be screened to avert late complications.

1.3.1.3 Management of gestational diabetes mellitus

In most pregnant women, GDM doesn't manifest itself. Most cases are only diagnosed when the blood sugar level is tested for the screening of GDM. In some women, the symptoms may appear when their blood glucose level gets too high (hyperglycaemia), such as polydipsia, polyuria, a dry mouth and tiredness. Although, some of these symptoms are common in pregnancy anyway and aren't necessarily a sign of a problem.

1.3.1.3.1 Screening

The National Institute for Clinical Excellence (NICE) recommends that screening should be offered at booking appointment to the healthy pregnant women with the following risk factors for GDM (**Table 1.2**).

Table 1.2. Current recommendations for screening (NICE Clinical Guidelines 2015).

Screening criteria according to NICE guidelines	
1.	BMI > 30/m^2
2.	Previous macrosomic baby weighing ≥ 4.5 kg
3.	Previous GDM
4.	First degree relative with diabetes (family history of diabetes)
5.	Family origin with a high incidence of diabetes (South Asian, dark Caribbean and Middle Eastern)

Offer women with any one of the above-mentioned risk factors testing for GDM. The use of 2-hour 75gm oral glucose tolerance test (OGTT) at 24-28 weeks is recommended to test for GDM in the women with risk factors.

Women with the history of GDM should be offered early days self-monitoring of blood glucose or a 2 hour 75gm oral glucose tolerance test (OGTT) as early as possible after booking i.e.; 16-18 weeks, followed by another OGTT (2 hour 75gm) at 24-28 weeks when the result of the 1st test is normal.

Beware of glycosuria ≥ 2+ one-time or ≥ 1+ two or more times detected by reagent strip testing during the routine antenatal care. It may indicate undiagnosed GDM. If this happens, consider further testing to exclude GDM (NICE Clinical Guideline 2015).

Ruling out the risk factor is controversial. Many experts (IADPSG) advise universal screening for all pregnant women (Metzger et al. 2010). Oral glucose tolerance test (OGTT) is extensively adopted throughout the world for the diagnosis of GDM because of its highest test performance, high sensitivity, high specificity and low false positive rates as compared to the other tests. After an overnight fast between 8 to 14 hours, OGTT is performed in the morning. The test is carried out to measure the glucose level by taking a blood sample at the beginning and 2-hour interval subsequently after drinking a solution that contains 75gm of glucose (NICE Clinical Guideline 2015).

1.3.1.3.2 Diagnosis

At least six types of different criteria have been practised globally for the diagnosis of GDM. The differences among the diagnostic tests are basically about the glucose load to be given, the timings and the type of blood sample (Agarwal et al. 2005; Agarwal et al. 2015). NICE recommends a 2-hour 75gm OGTT for the diagnosis of GDM (NICE Clinical Guideline 2015).

As per NICE guidelines, GDM diagnosed if the woman has either fasting plasma glucose level ≥ 5.6 mmol/l or a 2-hour 75gm OGTT plasma glucose level ≥ 7.8 mmol/l.

1.3.1.3.3 Management of GDM

Self-care is a very important component of the management of GDM. Both medical and nutrition treatments are required to be started once the GDM is diagnosed. The management regime suggested for GDM women includes dietary therapy, self-

monitoring of blood glucose levels and healthy lifestyle measures postpartum. The involvement of a dietician and the diabetes educator experienced in the care of GDM women facilitate these areas of management. All of the recommendations are described below.

1.3.1.3.3.1 Dietary therapy

It is generally accepted that the dietary therapy is a cornerstone of treatment of GDM. So, all the women with GDM should receive counselling from a specialist dietitian. Recommendations are individualised after a dietary assessment of each patient. The aim is to achieve normoglycaemia while providing the required nutrients for normal foetal growth and maternal health. Another aim (secondary) is to prevent excessive maternal weight gain, especially in women who are overweight/obese or have gained excess weight during pregnancy. Few trials have examined the efficacy of dietary therapy for GDM. Among those, a study has provided support for Medical Nutrition Therapy (MNT) for GDM as per the recommendations of American Diabetes Association (ADA). In this study, 215 GDM women were randomised to deliver either MNT or standard care. Only 24.6% of subjects in the MNT group required insulin (Reader 2007). The ADA proposes that all women should receive individualised counselling to provide sufficient calories and nutrients to meet the needs of gestation and consistency in the blood glucose goals. For obese women, a 30-33% calorie restriction to around 25 kcal/kg actual weight/day is recommended. Carbohydrate intake should be restricted to 35-40% of calories.

There are also data to support low carbohydrate diets in pregnancy, and for the carbohydrate to be of low glycemic index (GI). In a non-randomised study, GDM women on a diet comprising < 42% carbohydrates, had lower post-prandial blood glucose levels, were less likely to have need for insulin, and had a low incidence of large for gestational age (Major et al. 1998). A small study which randomised pregnant women to low GI or high GI diets found that the low GI group resulted in lower blood glucose levels, a blunting of the pregnancy allied rise in insulin resistance, and low birthweight (Clapp 2002). In another study of GI, women assigned to a low GI diet during pregnancy gave birth to infants who were lighter (3408 ± 78g vs. 3644 ± 90g) and had a low incidence of large for gestational age, as compared to the women given a high GI diet (Moses et al. 2006). GDM diagnosed women assigned to a low-

glycemic index diet regimen were observed to be considerably less likely to meet the criteria of insulin therapy. In the group of women with GDM allocated to a traditional high fibre and higher glycemic index diet, nearly half of the women who reached the criteria for insulin treatment avoided the treatment after shifting to the low-glycemic index diet (Moses et al. 2009).

As per above mention studies, it would be appropriate to recommend low GI carbohydrate diet to GDM women. Additional dietary measures are generally based upon the general recommendations for DM. A reduced intake of simple carbohydrates and fat are advisable. It is emphasised that the dietary intake should be spread over six meals daily including three main meals and three snacks, to avoid large carbohydrate loads at any time. Except for saccharin, which can cross the placenta and is therefore not recommended, other non-caloric sweeteners may be used in moderation (Cheung 2009).

1.3.1.3.3.2 Blood glucose monitoring

Women with GDM should perform self-monitoring of blood glucose (SMBG). Blood glucose levels are usually measured in the fasting state and 1-2 hours after meals. Management of post-prandial targets leads to superior pregnancy outcomes as compared to pre-prandial targets (de Veciana et al. 1995). Women performing SMBG showed 10% improvement in self-efficacy from baseline (Homko et al. 2002). According to a research study, pregnancy outcomes can be improved by meal based SMBG, and that available clinical evidence favours testing at 4-times a day in diet-treated GDM; e.g. before breakfast and 1 hour after each meal throughout the day (Jovanovic 2008).

The recommended treatment targets vary from country to country (**Table 1.3**). These are mostly consensus-based since the risk of complications is continuous and there are no clear thresholds above which the risk significantly increases. The initial intervention usually entails dietary advice, individualised if possible, and recommended by a dietitian. Lifestyle measures can deliver adequate control in the majority of subjects. If the blood glucose targets are not effectively met by lifestyle measures, and possibly a review of dietary intake, then pharmacotherapy should be considered. This generally means the commencement of insulin. Instead of basing the need for insulin on glycemic parameters only, some have supported the combined

usage of ultrasound assessment of foetal abdominal circumference together with blood glucose levels (Kjos et al. 2001). This practice allowed more women to avoid insulin therapy.

Table 1.3. Recommended glucose targets (Cheung 2009).

	Fasting (mmol/l)	**1 hr postprandial** (mmol/l)	**2 hr postprandial** (mmol/l)
NICE	5.3	7.8	6.4
ADA	5.8	8.6	7.2
ADIPS	5.5	8.0	7.0
CDA	5.3	7.8	6.7

1.3.1.3.3.3 Physical activity

In people with type II diabetes, there is sufficient evidence that regular physical activity enhances their insulin sensitivity, facilitates weight loss, and thus improves glucose control. Various small studies have tested whether regular exercise is also helpful in the management of GDM. In a study, 19 GDM women were randomised to either a regime of diet alone or diet with 20-minutes of supervised aerobic training three days/ week for six-weeks (Jovanovic-Peterson et al. 1989). The results of this modest amount of physical activity were reduced fasting glucose levels, low glucose response to a glucose challenge, and a lower HbA1c. In another study, 29 GDM women were randomised either to 30-minutes of exercise at 70% estimated maximal heart rate, 3-4 times/ week, or control (Avery et al. 1997). There was a trend to improved glucose levels in those who exercised, which did not reach significance. There were no differences in neonatal outcomes. Measures of cardiorespiratory fitness, however, improved. A study of 32 women randomised to circuit-type exercise 3-times a week or control, demonstrated that resistance training led to lower postprandial glucose levels along with a delay in the requirement of insulin (Brankston et al. 2004).

Amongst women with a pre-pregnancy BMI 25, those who exercised regularly were less likely to require insulin. Thereby, it appears quite reasonable to recommend that the GDM women with no medical or obstetric contraindication should maintain a practicable level of low and moderate intensity exercise throughout the pregnancy. The above-mentioned studies corroborate that moderate intensity physical activities such as; walking for 20-30 minutes each day, and presence at antenatal exercise classes can be safely encouraged, and that modest improvements in the glycemic control may be achieved.

1.3.1.3.3.4 Insulin therapy for gestational diabetes

When treatment targets are not attained by dietary means, at that time insulin is required. Prandial fast-acting insulin is given to control post-prandial hyperglycaemia, and bed-time basal insulin is administered if there is fasting hyperglycaemia. In some GDM women, an extra morning injection of basal insulin might further improve glycemic control. As the levels of insulin resistance vary between subjects, it is a common practice to administer small doses of insulin at the beginning, and after that to increase the doses at frequent intervals until target blood glucose levels are achieved. The required dose of insulin increases progressively over the last trimester of pregnancy. Frequent review and titration of the insulin dosage are recommended. Dissimilar to the situation of women with pre-existing type I diabetes, though, significant hypoglycaemia is uncommon in insulin-treated GDM women. Nevertheless, the women should be advised on appropriate hypoglycaemia prevention and management measures (Cheung 2009).

For long-time, fast-acting (regular) insulin, and intermediate-acting (isophane) insulin have been the most preferred insulins for the treatment of GDM. Human insulin does not usually cross the placenta, however; antibody bound animal insulin has been reported to do so (Menon et al. 1990). However, it has been shown by Jovanovic that it is maternal glucose control, rather than maternal anti-insulin antibody levels which influence birthweight (Jovanovic et al. 1992). Human insulin is considered safe in gestation as many years of experience have not suggested an increase in foetal complications as an outcome of its use. Nowadays, there is increasing evidence that the newer rapid-acting insulin analogues lispro and aspart are also safe during pregnancy, and therefore, they are commonly used. No increase in pregnancy

complications has been found in observational studies where lispro was used, in either woman with GDM or pre-existing diabetes (Bhattacharyya et al. 2001; Persson et al. 2002; Aydin et al. 2008).

There is a report regarding the use of aspart in pregnancy suggesting that aspart is as safe and effective as human insulin (Hod et al. 2008). With respect to GDM, there have been several small randomised studies comparing the use of rapid-acting insulin analogues with the regular insulin. They all have demonstrated that the rapid-acting analogues are as efficacious as regular insulin in the treatment of GDM, with parallel, if not favourable outcomes (Cheung 2009). Data regarding the long-acting insulin analogues are less clear than for rapid-acting analogues. Concerns have been stated about the usage of glargine in pregnancy, as of its potential effect on mitogenesis (Hirsch 2005; Jovanovic and Pettitt 2007).

NICE recommends offering immediate treatment with insulin (with or without metformin), along with changes in diet and exercise to GDM women with fasting plasma glucose level of 7.0 mmol/l or above at the time of diagnosis (NICE Clinical Guideline 2015).

1.3.1.3.3.5 Oral antidiabetic agents in GDM

There is a controversy regarding the use of oral hypoglycemic agents in pregnancy. Many government drug-agencies have not validated their use during pregnancy, and major specialty diabetes organisations suggest that oral agents be stopped if the woman had been taking them pre-pregnancy (McElduff et al. 2005; Canadian Diabetes Association 2008; Kitzmiller et al. 2008).

Though, insulin is considered as the gold standard for the treatment of hyperglycaemia in pregnancy when dietary and lifestyle modifications cannot achieve the recommended glucose targets. However, recent studies have shown that certain oral antidiabetic drugs may be safe and acceptable alternatives (Kalra et al. 2015; Singh and Singh 2015). NICE recommends offering metformin to women with GDM (fasting plasma glucose < 7.0 mmol/l at diagnosis) not meeting blood glucose targets with diet and exercise within one to two weeks. The GDM women in whom blood glucose targets are not achieved with metformin but who decline insulin treatment or

who cannot tolerate metformin can be considered for glibenclamide (NICE Clinical Guideline 2015).

1.3.1.4 Future risks of GDM

1.3.1.4.1 Increased maternal risks

Pre-eclampsia and the gestational hypertension are apparently more common in women with GDM (Dukler et al. 2001; Innes et al. 2001; Xiong et al. 2001). Preeclampsia increases the incidence of (3-fold increased) preterm delivery (Vatten and Skjaerven 2004). Caesarian section is likely to be carried out in females with GDM and labour is required to be induced (Aberg et al. 2001; Brody et al. 2003; Berg et al. 2007). Preeclampsia is particularly developed more in pregnant obese females with insulin-treated GDM having poor glycemic control in comparison to pregnant obese females with well-controlled insulin-treated GDM. Chronic hypertension is two to three folds more prevalent in overweight and obese GDM females irrespective of the treatment or glycemic control level (Langer et al. 2005).

The increased risk of developing DM later in life for GDM women is well known. It has been also recognised that GDM women have up to 8-fold higher risk of developing metabolic syndrome (MS). This syndrome is linked to a high rate of type II diabetes and cardiovascular complications (Peters et al. 1996; Kim et al. 2002). The collective incidence of type II ranges from 2.6% to above 70% at six weeks to 28 years post-partum in females with prior GDM (Kim et al. 2002).

1.3.1.4.2 Increased child risks

GDM during pregnancy possibly affects the outcome of the progenies. A higher percentage of children are born premature, macrosomic and have dystocia, (Jensen et al. 2000; Yang et al. 2002; Ostlund et al. 2003), even when the GDM is being treated (Bartha et al. 2003). Hypoglycaemia affects newborn babies more frequently, and they require care in neonatal intensive care units (NICU) to a greater degree (Simmons et al. 2000; Svare et al. 2001). It is revealed that if GDM is diagnosed in the third trimester, the risks of congenital anomalies are reduced (Savona-Ventura and Gatt 2004). Some specific anomalies are reported more frequently amongst GDM affected pregnancies, for example, certain cardiac defects, spinal deformations and oesophageal /intestinal atresia (Aberg et al. 2001).

It has become progressively clear over the past decades that many foetal stresses may lead to foetal programming and alteration in the normal developmental gene expression pattern. Current research indicates that the children of the diabetic mother remain at high risk for a variety of developmental disturbances: obesity (Dabelea and Pettitt 2001; Brody et al. 2003; Gillman et al. 2003), impaired glucose tolerance or diabetes (Silverman et al. 1995; Brody et al. 2003) and diminished neurobehavioral capacities (Rizzo et al. 1991; Sells et al. 1994; Lincoln et al. 1996). It is also observed that LGA offspring of GDM mothers had a higher than a 3-fold risk for symptoms associated with metabolic syndrome, at 11 years of age (Boney et al. 2005). Females with previous GDM reported that their children to be less healthy as compared to the controls 3 to 5 years after childbirth (Feig et al. 1998). Also, children of the mothers who had DM or GDM during pregnancy reported lower marks in school (Dahlquist and Kallen 2007). Thus, it would be sensible to speculate that the process whereby a stimulus or insult (glucose toxicity and other metabolic fuels) acting at a critical stage of development in early and during late intrauterine life, may alter gene expression patterns for life.

1.4 Pregnancy, oxidative stress and micronutrients

1.4.1 Overview

The significance of appropriate nutrition consumed prior and during pregnancy on optimising the health status of mother and infant is known for long (Fawzi et al. 2007; Allen et al. 2009). “Micronutrients” is a term used collectively for minerals and vitamins. These dietary components are required in traces for the maintenance of normal physiological functions of human body. They are essential dietary elements, as the body cannot synthesise them and their inadequacy results in specific metabolic deformities especially in children, pregnant and lactating women. High Systemic oxidative stress occurs late in pregnancy because of increased metabolic demand, which leads to decreased availability of antioxidants during pregnancy. Therefore, the content of these micronutrients should be increased in the diet in order to compensate their substantial loss (Bedaiwy et al. 2003; Oyawoye et al. 2003; Berchieri et al. 2011). Insufficient supplies of micronutrients throughout pregnancy can have unfavourable impacts on the mother, for example, anaemia, hypertension, labour complications or even death. The foetus can also be affected, such as stillbirth, preterm delivery, intrauterine growth retardation, congenital malformations, reduced immunity and organ abnormalities. Hence, attention should be paid to maintain adequate levels of micronutrients throughout the gestation **(**King 2000; Owens and Fall 2008).

1.4.2 Oxidative stress

Oxidative stress is explained as an imbalance between the production of reactive oxygen species and antioxidant defences, characterised by increased concentration of oxygen and non-oxygen-derived products that stimulate critical and sometimes irreversible cell injury (Caimi et al. 2003). These reactive species are generated as by-products of metabolism, physiological mediators and signalling molecules (Evans and Halliwell 2001). These reactive species include reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chloride species (RCS). These reactive oxygen species are produced endogenously under both physiologic (pregnancy) and pathologic conditions (Cotovio et al. 2001). They are also known as free radicals (highly reactive molecules that damage cells throughout the body). They are believed to be responsible for cardiovascular diseases, cancer, neurological disorders, cataract, arthritis, ageing, fatigue, and muscle damage (Food and Nutrition

Board 2000; Fang et al. 2002). During pregnancy, the generation of lipoperoxides exceeds their decomposition rate creating oxidative stress. These lipoperoxides also increase in the foetus as it grows but to a lesser degree than of mother (Sobki et al. 2004). This increased oxidative stress causes lipid and protein peroxidation, which impairs endothelial cell function (Serdar et al. 2003). It can affect the foetal skeletal formation, and it can also cause spontaneous abortion, recurrent pregnancy loss and preeclampsia in pregnancy (Prater et al. 2008; Agarwal et al. 2012).

1.4.3 Antioxidants

Defence system of the human body against free radicals is a system of antioxidant molecules that interact rapidly with free radicals and stop the chain reaction before it can destroy vital macromolecules (proteins, nucleic acids, starches and lipids). Antioxidant defence system includes both enzymatic (SOD, catalase, GSH peroxidase and GSH reductase) and non-enzymatic components (mainly vitamin E, A, C and β-carotene), (Cho and Choi 1994).

Vitamin E, A and β-carotene are low molecular antioxidant compounds. They absorb into the blood by the lymphatic system and then reach the liver and fatty tissues by the blood where they are stored for their required actions (Grzelinska et al. 2007). Vitamin E is the main liposoluble antioxidant in human body which scavenges peroxyl radicals produced during lipid peroxidation by transferring its phenolic hydrogen to a peroxyl free radical of a peroxidized polyunsaturated fat (PUFA), thus breaking the radical chain reaction and preventing the peroxidation of PUFA in cellular and subcellular membrane phospholipids (Oostenbrug et al. 1997; Grissa et al. 2007)**.** This lipid soluble property permits it to diffuse into various tissues rapidly. Vitamin A is another non-enzymatic antioxidant capable of reacting directly with free radicals while β-carotene is reported to scavenge singlet molecular oxygen effectively (Baydas et al. 2002).

1.4.3.1 Vitamin A, β-carotene and vitamin E

Vitamin A is a generic term applied to a large number of closely related compounds. Chemically, it is a poly-isoprenoid compound with a fat-soluble property and also serves as a precursor for the synthesis of some hormones. There are two active and major forms of vitamin A; retinol (vitamin A1) and 3-dehydroretinol

(vitamin A2). A chemically diverse group of plant carotenoids forms provitamin A through which vitamin A is synthesised enzymatically in the walls of the small intestine and liver. β-carotene is the most active carotene among over 50 carotenoids, and it is responsible for the biological action of vitamin A, directly or after its transformation into two molecules of this vitamin (Cikot et al. 2001).

Vitamin E is another vital fat-soluble vitamin that occurs naturally. It is a collective name for a family of eight antioxidants; four tocopherols (α β, γ and δ) and four tocotrienols (α β, γ and δ), which perform similar activities (Nelson and Cox 2005; Traber 2007). Out of the four types, α-tocopherol (5,7,8-trimethyl tocol) is the most vital type of vitamin E. Its levels are maintained actively in the body; hence, it is the type of vitamin E present excessively in the blood and tissues (**Figure 1.12a, b, c**), (Ogbodo et al. 2012).

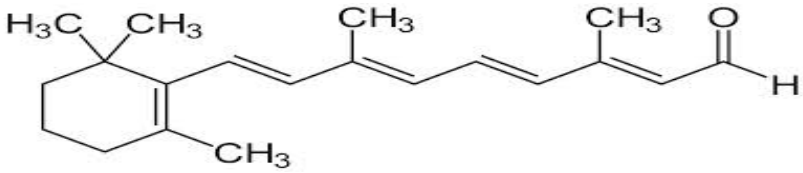

Figure 1.12a. Structure of retinol (adopted from (Ogbodo et al. 2012).

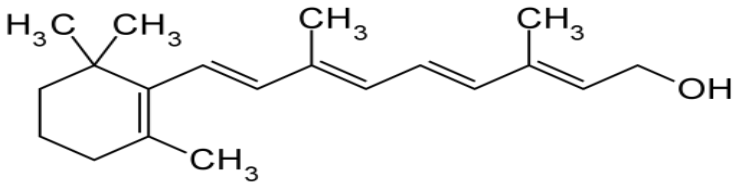

Figure 1.12b. Structure of β-carotene (adopted from http://www.thecollapsedwavefunction.com).

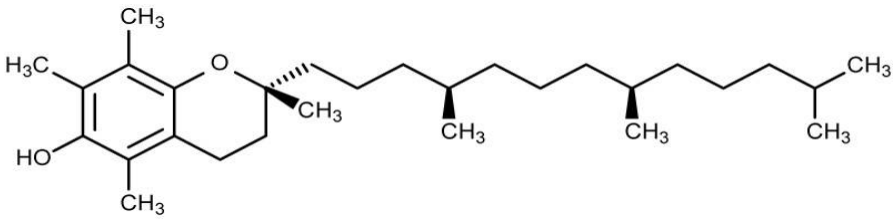

Figure 1.12c. Structure of alpha-tocopherol (adopted from (Ogbodo et al. 2012).

1.4.3.1.1 Sources

Animal products, vegetables and yellow-pigmented plant products are the various sources of vitamin A. Fish liver oil, liver, eggs, milk and butter are major animal sources while the yellow pigmented items including carrots, sweet potatoes and other yellow vegetables contain β-carotene (Nelson and Cox 2005). Vitamin E was initially isolated from wheat germ oil where it is found in abundance. It is also found in cottonseed oil, rice, eggs and vegetable oils (Cohn 1975).

1.4.3.1.2 Functions

Vitamin A and β-carotene are vital for normal vision, tissue growth, building up epithelium and skin, and stabilization of epithelial cells (Maden 2000; Smith and Steinemann 2000), maintenance of normal neural shields, synthesis of adrenal cortex hormones, immune reactions, thyroid thyroxine secretion, synthesis of erythrocytes and defense against cancer development (Sies and Stahl 1995). Vitamin E averts oxidative damage to cellular structures and tissues by breaking chain reaction of free radicals so participating in the maintenance of an appropriate cell membrane structure, inhibiting the generation of micro-clots and thus blocking the production of nitrosamines (Malila et al. 2002; Feskanich et al. 2003, Osganian et al. 2003; Knekt et al. 2004). Vitamin E is the most significant micronutrient involved in the protection of LDL from oxidation and increasing mean HDL level while decreasing LDL level (Kagan et al. 1992; Paolisso et al. 1993). Numerous studies indicate that adequate plasma concentrations of vitamin E and β-carotene inhibit oxidation of low-density lipo-proteins, thus decreasing the risk for the development of atherosclerosis,

cardiovascular diseases, cancers and age-related macular degeneration (Pryor 2000; De Wart et al. 2001; Rao 2002).

1.4.3.1.3 Requirements

The latest recommended dietary allowance (RDA) is based on the amount needed to ensure adequate stores of the vitamin in the body to support reproductive, immune function, gene expression and vision (**Table 1.4**). A tabular presentation of RDA is given below.

Table 1.4. RDA for vitamin A and E (adopted from Food and Nutrition Board 2000; 2001).

Group	Age	RDA of vitamin A	RDA of vitamin E
Infants	0-6 months	400 µg/day	4 mg/day
	7-12 months	500 µg/day	5 mg/day
Adults	14 years and above (vitamin RDA)	900 µg/day (male)	----------
		700 µg/day (female)	
	19 years and above (vitamin E RDA)	----------	15 mg/day
Pregnancy	18 years and below	750 µg/day	15 mg/day (for pregnancy all ages)
	19 years and above	770 µg/day	

1.4.4 Antioxidant status during normal pregnancy

There is a less number of studies dealing with the status of vitamin E, A and β-carotene in both cord and maternal blood during normal pregnancy. Baydas et al. (2002) have clearly demonstrated that vitamin E levels in cord blood were lower than in maternal blood. This finding corroborates previous reports (Chen et al. 1996; Chan et al. 1999; Kiely et al. 1999). A positive correlation between the cord and maternal serum levels of vitamin E as determined in this study was also verified by other investigators (Vobecky et al. 1982; Shah et al. 1987). It is obvious that neonatal vitamin E concentration is dependent on maternal vitamin E levels and it is well recognised that placental transfer of vitamin E is limited (Specker et al. 1992). Nonetheless, the mechanism of transportation is still unclear.

Vitamin E concentrations during pregnancy progressively increased compared with preconception values. This finding is following previous reports (Mooij et al. 1993). The increase in vitamin E might have a compensatory effect on the increase in oxygen radical formation as gestation progresses (Wang et al. 1991). The elevation of vitamin E seems to correspond with the increase in products of membrane damage during late gestation. The concentration of retinol minor decreased during pregnancy. In contrast, the concentration of α-tocopherol showed a progressive increased or showed only minor changes during pregnancy, when compared with preconception values. Most obvious vitamin changes occur gradually during pregnancy, but its mechanism is not completely understood yet. It may be due to increase renal excretion, hormonal influences and haemodilution occurring during gestation. Another reason for decreased levels of vitamins may be the increasing requirement of vitamins during pregnancy both for mother and foetus (Cikot et al. 2001).

1.4.5 Antioxidant status during GDM

Along with several other complications, GDM mothers and their foetus are also exposed to oxidative stress associated with down-regulation of antioxidant status, resulting in the production of highly reactive oxygen free radicals. These free radicals are highly toxic to the cells, particularly in the plasma membranes where they interact with the lipid bilayer (Cederberg et al. 2001; Damasceno et al. 2002; White et al. 2002; Prasenjit et al. 2008). GDM is the most frequent metabolic disorder of pregnancy occurring in 1-10% of all pregnancies with increased susceptibility to oxidative stress

(Dey et al. 2008), affecting even the foetuses and can put the foetus at the risk of developing congenital malformations, premature birth or other serious complications (Sobki et al. 2004). The reason for developing oxidative stress is established as down regulation of antioxidant status and disturbance in free radical scavenging system in DM (Chen and Scholl 2005; Grissa et al. 2007). Another reason for this elevated oxidative stress was found to be impaired SOD activities and enhanced levels of thiobarbituric acid-reactive substances (TBARSs) during GDM. In animal models, experimental diabetes disrupts maternal and foetal lipid metabolism. It was also found that there was a significant decrease in plasma arachidonic acid and an increase in linoleic acid levels in diabetic pregnant rats. This may be because of impaired action of desaturases enzymes. This diabetes-induced low concentration of plasma AA has a critical role in maintaining the appropriate mass and function of beta cells especially by affecting rates of cell proliferation and insulin secretion (Yessoufou and Moutairou 2011).

In another study, a significant depletion of vitamin E in cord plasma of GDM patients was found which is in concurrence with other studies and is suggestive of raised oxidative stress. A slight reduction in vitamin E levels in maternal plasma of GDM patient is also supported by findings of another study (Kharb 2000), but it is in contrast to the study of Bates et al. (1997) who has shown an increase in serum vitamin E levels in diabetic pregnant women. This decrease of vitamin E levels in the cord blood may be due to the progressive utilisation of this vitamin during excessive lipid peroxidation. Alternatively, these lower cord vitamin E levels in GDM patients may be due to its preferential mobilisation toward the tissues with the maximum requirement. Another study revealed that novel alpha-tocopherol binding protein in the human placenta may control the movement of tocopherols between the foetus and mother. It was further observed that tocopherol ratios in the cord or maternal plasma of control group were significantly higher than that of the GDM patients regardless of the treatment they received. However, this ratio was always higher in maternal plasma than cord plasma (Sobki et al. 2004). Some other researchers found that GDM women exhibit decreased levels of vitamin E without any changes in vitamin A (Yessoufou and Moutairou 2011). Investigations by the researchers also support above-mentioned findings (Peuchant et al. 2004; Grissa et al. 2007).

Specific Aims:

The aims of this study are to investigate if;

- Diabetes in pregnancy compromises plasma omega-3 and omega-6 fatty acid status of pregnant women at diagnosis.
- Babies born to diabetic women have reduced levels of plasma omega-3 and omega-6 fatty acids at birth.
- High pre-pregnancy body mass index alters the plasma fatty acid profile in GDM women.
- Supplementation with the omega-3 fatty acid, docosahexaenoic, enhances the level of the aforementioned fatty acid in plasma of gestational diabetic mothers and their neonates at delivery.
- Docosahexaenoic acid supplementation improves the plasma antioxidant status of gestational diabetic women and their neonates at birth.

Null Hypothesis:

- Diabetes in pregnancy does not compromise plasma omega-3 and omega-6 fatty acid status of women affected with the condition at diagnosis.
- Babies born to diabetic mothers do not have reduced levels of plasma omega-3 and omega-6 fatty acids at birth.
- High pre-pregnancy body mass index does not alter the plasma fatty acid profile in GDM women.
- Supplementation with docosahexaenoic acid during pregnancy does not enhance the level of the fatty acid in plasma of pregnant women affected with diabetes and their neonates at birth.
- Docosahexaenoic acid supplementation of pregnant women with diabetes does not improve the plasma antioxidant status of mothers and their neonates at delivery.

CHAPTER 2

Subjects and methods----------(59-79)

2.1 Subjects and recruitment criteria

A total of 170 women (between ages 17 - 45 years) with single pregnancy (healthy/non-diabetic n = 28 and gestational diabetic women, GDM n = 142) were recruited (17^{th} - 34^{th} weeks of gestation) during their visit to the antenatal or diabetic clinics at Newham University Hospital, London, UK. The following NICE criterion was adopted for the screening of gestational diabetes.

2.1.1 Diagnosis of gestational diabetes mellitus

After an overnight fast, high risk identified women undergone a 75gm oral glucose tolerance test (OGTT) and their blood glucose level were monitored at 0 and 120 minutes. GDM is diagnosed when the blood plasma glucose concentration at 0 min (fasting) ≥ 5.6mmol/l and/or 120 min (non-fasting) ≥ 7.8mmol/l (NICE Clinical Guidelines 2008; NICE Clinical Guidelines 2015).

2.1.2 Exclusion criteria

Women who had been taking omega-3 supplements during the period of time leading to their current pregnancy were excluded. Those who were on or planning to receive tocolytic or corticosteroid therapy were also excluded.

Women with chronic medical conditions such as; HIV/AIDS, kidney disease, congenital heart disease, autoimmune disease (e.g. lupus or anti-phospholipid syndrome), and haematological disease (e.g. sickle cell disease or other hemoglobinopathies) were also excluded.

2.1.3 Ethical approval and consent

Ethical approval for the study was obtained from the East London and The City HA Local Research Ethics Committee 3 (REC Reference Number; 06/Q0605/89) and registered with ISRCTN Register (registration no. ISRCTN68997518). A written informed consent was taken from all the participants of the study. All the investigators, midwives and participants were blinded to the allocation of the intervention till the completion of the analysis and the data recording.

2.1.4 Study design and treatment

The study was a randomised, double-blind, placebo-controlled trial. After recruitment, the healthy pregnant and GDM women were randomly assigned to the active treatment group (ω-3 enriched supplement) or placebo group. The active treatment group (n = 82) received two omega-3 capsules daily and the placebo group (n = 88) received two placebo capsules daily, until delivery. Each omega-3 capsule provided 300mg of DHA, 42mg of EPA and 8.4mg of AA. Whereas, placebo capsule contained high oleic acid sunflower seed oil (721mg of oleic acid). 8µg of α-tocopherol (vitamin E) was added as an antioxidant in both supplements. These two supplements were filled in identical soft gelatin, oblong shaped capsules (750mg in size). Randomization was done using a random code generated by the supplement supplier (Equazen/Vifor Pharma Ltd., Switzerland).

2.1.5 Sample size

Sample size was calculated based on previous case control study conducted by our research group. A difference of 35% was found in DHA between neonates of healthy pregnant women (5.4%) and GDM women (4.0%). It was ssumed that supplementation of GDM women with DHA-enriched formula will increase the level of the fatty acid in the neonates of the GDM to that of the neonates of healthy pregnant women. The result of this calcualtion indicated that 80 women (40 in each arm of the study) will be required to detect changes in the level of the fatty acid with 80% power at 5% significance level. The sample size was calcualted using G*Power 3 (Faul et al. 2007) and based on two independent groups, two-tailed t-test with an alpha of 0.05.

2.1.6 Demographic and obstetric information

The details of demographic, clinical and obtetric data were collected from the hospital medical records.

2.1.7 Neonatal anthropometric measurement

Weight and length of the neonates were recorded at delivery. It was taken by the midwife who attended the delivery as a part of routine practice. Head, shoulder, mid-arm and abdominal circumferences were measured by research midwives (JH and

IN), using Seca 210 portable measuring mat and Seca 201 ergonomic circumference measuring tape (Seca UK, Birmingham, UK).

2.1.8 Biological specimen

A volume of about 5-10ml of non-fasting venous blood was obtained from both the placebo and active-treatment groups. Blood samples were collected at 2-time points, first at recruitment (from mum) and second at delivery (from both mum and cord), for analysis of fatty acids and antioxidant vitamins. At delivery, venous blood was taken from the placental side of clamped cord.

2.1.9 Sample collection and preparation of plasma

The whole blood was collected into the vacutainer tubes containing an anticoagulant, ethylenediaminetetraacetic acid (EDTA, K2 (spray-dried), BD Company) at Newham General Hospital. The blood samples collected during the day were immediately transported to Lipidomics and Nutrition Research Centre (LNRC) laboratory, London Metropolitan University. Where these samples were instantly separated into plasma and red cells by cold centrifugation at 3000 rpm for 15 minutes. After centrifugation, the top plasma layer was removed into clearly labelled eppendorf tube, closed tightly under a stream of oxygen-free nitrogen (OFN) and stored at -70^{o}C until further analysis. Subsequently, the bottom erythrocytes pellet was recovered by aspiring the supernatant and made the volume up to 10ml with PBS (physiological saline, 0.85% NaCl). These tubes were then vortex and inverted gently to re-suspend the cells and centrifuged at 3000rpm for 5 minutes. The supernatant was discarded, and the same procedure was repeated again (total 2 washings with PBS, vortex, centrifuge and aspiration of supernatant) to remove traces of plasma and buffy coat. Finally, the erythrocytes were transferred into clearly labelled eppendorf tube, closed tightly under a stream of OFN. The blood samples collected at night time or over the weekend were processed at pathology laboratory, Newham General Hospital and then subsequently sent to the LNRC, London Metropolitan University on next working day. All the samples were stored at -70^{o}C until further analysis (**Figure 2.1**).

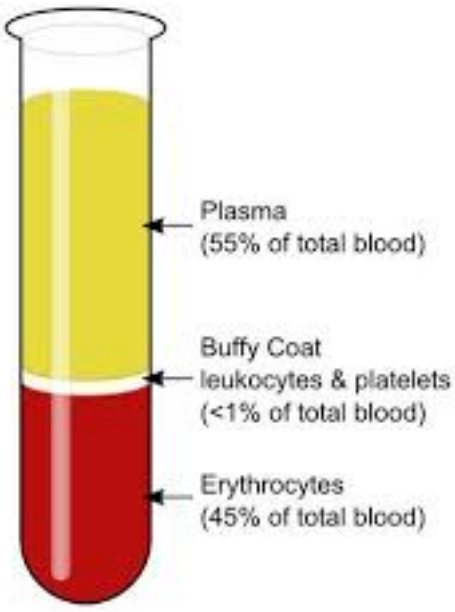

Figure 2.1. Diagrammatic representation of separation of blood cells (adopted from https://en.wikipedia.org/wiki/File:Blood-centrifugation-scheme.png).

2.2 Plasma fatty acids analysis

2.2.1 Extraction of plasma total lipids

Plasma total lipids were extracted based on the method of Folch et al. (1957) by homogenising the samples in chloroform and methanol, (C/M, 2:1, v/v). 1ml of defrosted plasma was homogenised with 45ml of C/M (chloroform/methanol, 30+15ml, + BHT 0.01% w/v) in a 100ml extraction tube. Homogenization was done by vortex the sample with solvents thoroughly for two to three minutes and then flushed with oxygen-free nitrogen (OFN) approximately for one minute by bubbling gas throughout the sample (to displace any dissolved oxygen). The extraction tube was capped instantly and stored at 4^{o}C in a refrigerator for 24 hours. All the solvents used, contained butylated hydroxyl toluene (BHT) as an antioxidant to prevent potential oxidation (possible damage to the extract due to oxygen caused by free radicals) of unsaturated fatty acids. Also, all the samples were handled under OFN (Folch et al. 1957). The used chemicals and their sources are enlisted in detail in **Appendix 2**.

2.2.2 Separation of non-lipid impurities from lipids "Partitioning"

The tubes containing extracted plasma samples were removed from the refrigerator and allowed to stand under dim light, at room temperature, for 30 minutes in order to prevent condensation. The extracted samples were then filtered into 100ml separating funnels, through the conical metal funnels lined by Grade 1 filter papers (Whatman International Ltd. England). The residue in the extraction vessel and the filter paper (lining the conical metal funnels) was washed with 10ml + 5ml of C/M (2:1 v/v, + BHT) just to ensure almost complete transfer of whole lipid contents (**Figure 2.2**).

After complete filtration, the filter funnels along with filter papers were removed and a volume of 15ml of saline (85% w/v of NaCl, equivalent to 25% v/v of the total filtrate volume) was added to the filtrate (in separating funnels) for the purpose of complete phase separation. The samples were then flushed thoroughly by bubbling OFN and stored at 4^{o}C in a refrigerator overnight.

Figure 2.2. Separating funnels used for partitioning (picture taken at LNRC lab).

2.2.3 Rotary evaporation

On the next day, the separating funnels were removed from the refrigerator and allowed to stand under dim light, at room temperature for 30 minutes to attain equilibrium. The lower organic layer containing purified lipids (organic) was then drained into a 100ml round bottom flask. Afterwards, the solvent was removed by the rotary evaporator (vacuum pump V-700, Buchi, Switzerland) under reduced pressure in a water bath at 37^{o}C. The lipid residue-containing flask was rinsed twice with 2ml of methanol just to ensure the complete removal of any residual water in the sample. After absolute drying, the whole lipid extract was transferred to a 10ml glass vial by washing thrice with 2ml of C/M (2:1 v/v + 0.01% BHT w/v). This total 6ml volume of the recovered lipids was then reduced to 1ml under a stream of OFN at 37^{o}C, flushed carefully and stored at -20^{o}C until required for thin-layer chromatography (TLC), (**Figure 2.3**).

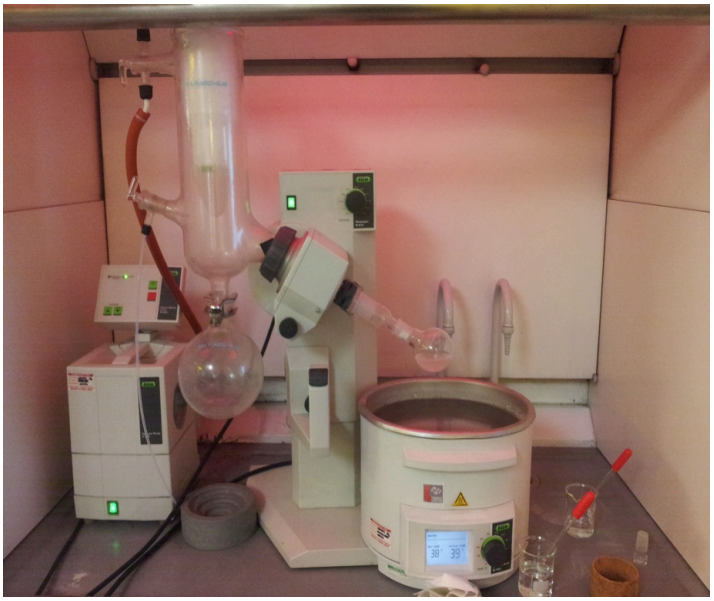

Figure 2.3. Recovery of total lipids by rotary evaporator (picture taken at LNRC lab).

2.2.4 Thin-layer chromatography (TLC)

2.2.4.1 Preparation and activation of TLC plates

For consistent results, commercially available 20×20 cm, pre-coated silica gel glass plates (0.25 mm thickness) were used for analysis (Merck KGa, Germany); however, TLC plates can be prepared manually in the laboratory. For analytical purposes, layers of adsorbent 0.25mm thick or less give maximum resolution. Glass is by far the most commonly used support for the adsorbent in the TLC of lipids. It is resistant to acid and alkalis used in developing solvents or detection sprays (Hinrichsen and Nas 2006). TLC plates were scored about 2.5cm from the top (to prevent solvent over-elution) and 0.5 cm from each side. Each plate was scored from right to left into three equal channels (6×6×6), by scraping the narrow lines of adsorbent. So, there were three compartments (for each sample) on each plate. The purpose of channelling was to ensure the sample's application at defined regular distance to prevent the spread of one sample into another. For good separation by silica gel, the water content of gel must be carefully controlled. Therefore, after complete scoring, the TLC plates were conditioned for 1 hour at 110-120^{o}C in an oven just to ensure its dryness (due to reduced activity of adsorbent in the presence of water) and stored in a desiccator until the time of the sample application (**Appendix 2**).

2.2.4.2 Solvent system

The solvent mixture/ mobile phases; **c**hloroform: methanol: methylamine (C:M:M, 65:35:15 v/v/v + 0.01% BHT w/v), and petroleum spirit: ether: formic acid: methanol (P:E:F:M, 85:15:2.5:1 v/v + 0.01% BHT w/v) were prepared freshly to separate the phospholipids (choline phosphoglycerides, CPG) and neutral lipid (cholesterol ester, CE and Triglyceride, TG) fractions respectively. Each mobile phase was mixed thoroughly. The total neutral lipids were further fractionated with later mobile phase after recovery from the TLC plate containing polar and non-polar lipids separately (**Appendix 2**).

2.2.4.3 Developing chambers

For the development of 20×20 plates, rectangular glass chambers of 10×21×21cm approximate dimensions are commonly used. In addition to being inert to the developing solvents, glass is transparent and allows the progress of the

ascending solvents to be monitored. Classical chambers have flat bottoms and sides and allow a maximum of two plates to be developed simultaneously.

A freshly prepared 150ml of mobile phase (C:M:M for phospholipids and P:E:F:M for neutral lipids) was poured into the TLC tank lined with filter papers. The tank was lined with filter paper and capped immediately with a well-fitted heavy glass lid to seal the environment inside and helped to saturate the atmosphere with solvent vapour that speeds up the analysis. The developing tank was closed for almost 30 minutes to achieve equilibrium before use.

2.2.4.4 Application of samples on TLC plates

The quality of lipids analysis by TLC depends greatly on the careful application of the sample mixture to the adsorbent. Care was taken not to damage the adsorbent layer during sample application and TLC plates were only handled by the edges, to avoid contamination from fingers. The activated plate was kept smoothly on a balanced surface lined with filter paper, in dim light. A led pencil was used lightly to draw a narrow line almost 2cm from the bottom edge. This application line was used as a locator on which the sample was loaded. The sample was dried under OFN and total lipid extract was re-dissolved thrice with 10, 8 and 6 drops of C/M (2:1 v/v +0.01% BHT w/v) respectively, for its complete and uniform application on TLC plate. The sample was applied as a narrow uniform streak along the application line using a disposable capillary tube (75µl micro-haematocrit). A maximum number of three samples were loaded on each plate. Samples were identified by their allocated numbers written by a soft pencil at the top of the plate (before each sample), (**Figure 2.4**).

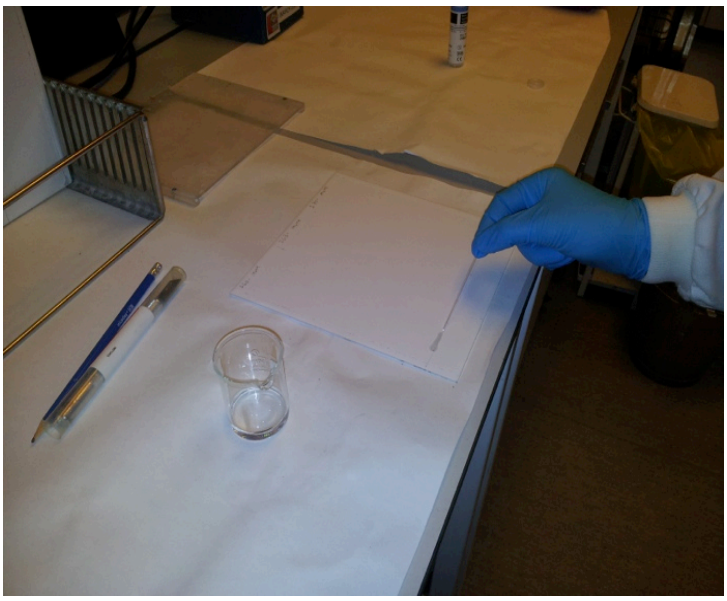

Figure 2.4. Application of total lipid extract on a TLC plate by micro-haematocrit tube (picture taken at LNRC lab).

2.2.4.5 Development of TLC plates

With least disturbance of atmospheric equilibrium inside, the two plates were transferred quickly (facing each other) to the developing tank containing mobile phase. The tank was covered with a lid instantly by ensuring a good seal. The solvent moved up the plate by the capillary action taking the various components with it at different rates, according to the extent to which they were held by the adsorbent. When the solvent front was travelled across the plates and touched the line scored at the top of the plates, the plates were kept further inside the tank for 5-10 minutes to improve the definition of bands. The time taken for a TLC plate to develop depends on the solvent system employed. For instance, a TLC plate takes much longer time to develop in a polar mobile phase as compared to be in non-polar. Phospholipids plates took nearly 90 minutes to develop while the neutral lipids plates took about 30 minutes.

2.2.4.6 Visualization of TLC plates

After removal from the tank, the developed plates were air-dried to remove the solvent and sprayed with a reagent (methanolic solution of 2, 7-dichlorofluorescein, 0.1%, w/v) until each plate was coated lightly and uniformly. Since lipids are generally colourless, the separated lipid components have to be rendered visible by chemical reagents. The chemical reagent spray can be specific for certain types of lipid or certain functional groups, or it may be a non-specific reagent for all lipids. Therefore, a methanolic solution of 2, 7-dichlorofluorescein is most frequently used spray that renders all lipids visible and causes lipids to show up as bright yellow spots/bands on a yellow/green background under UV light (Vinson and Hooyman 1977).

After spray, the plates were air-dried again for few minutes to reduce the background staining on view. The entire procedure was carried out in a dark fume cupboard. Successively, the phospholipids (CPG on phospholipid's plate) and neutral lipid's bands (CE and TG on neutral lipid plate) were visualized under ultraviolet light (UV, 533nm, a non-destructive visualization method). These lipid fractions were appeared as lines of discrete bands. Each band was then marked carefully with a soft pencil and identified by using commercially available authentic standards (**Appendices 5, 6, Figure 2.5**).

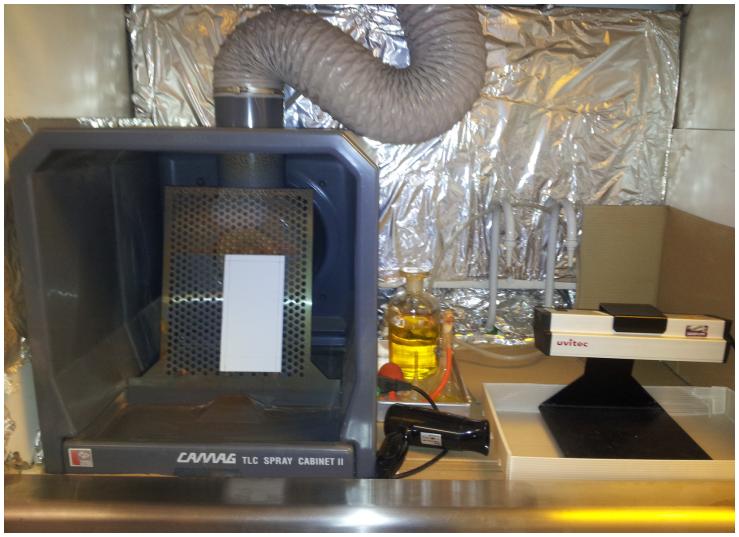

Figure 2.5. TLC spray cabinet and UV light source used for staining and visualization of the TLC plates (picture taken at LNRC lab).

2.2.4.7 Scraping of lipid bands

The required bands (plasma CPG and neutral lipids) were recovered after detaching by an appropriate non-destructive method. A blunt spatula was used to detach the adsorbent band onto a filter paper, from both the plasma phospholipids and neutral lipids containing plates and transferred the flakes and dust to their labelled methylating tubes (16×160 mm Pyrex tube fitted with a PTFE lined screw top, Fisher Scientific, UK).

2.2.5 Separation /recovery of neutral lipids

Lipids mostly recover by repeatedly mixing the adsorbent with the solvent in a test tube, centrifuging and decanting the supernatant liquid. The non-polar neutral lipids (NL) travel in the polar solvent system collectively as one band rather than separating into different fractions. After elution, the NL bands were reached at the top of the TLC plates, scraped by the scraper and transferred to a labelled centrifuge tube. Flakes and dust were suspended in 5ml petroleum spirit: diethyl ether (P: E, 50:50 v/v, 0.01% BHT w/v) shook well and centrifuged at 3000 rpm for 5 minutes. The supernatant containing the neutral lipids was transferred to a labelled glass vial of 10ml. The whole step was repeated again. In the end, each glass vial had a 10ml volume of supernatant in total. These recovered NLs were concentrated under a stream of OFN and loaded on a TLC plate in the same manner as described above, by dissolving in 1ml of C/M (2:1 v/v +0.01% BHT w/v). A mixture of petroleum spirit: ether: formic acid: methanol (P:E:F:M, 85:15:2.5:1 v/v + 0.01% BHT w/v) was used as mobile phase. Two bands (TG and CE) were scraped off into the methylating tubes. The rest of the procedure was same as described above in TLC protocol.

2.2.6 Preparation of fatty acid methyl ester “FAME”

Before the fatty acid composition of a lipid can be determined by gas chromatography, it is necessary to prepare the comparatively volatile methyl ester derivatives of the fatty acid components. This must be by far the most common chemical reaction performed by lipid analysts.

2.2.6.1 "Trans-methylation" (acid-catalysed trans-esterification)

Fatty acid components require to be converted into fatty acid methyl esters so as to improve their volatility and hence to ensure better gas chromatographic peak shape (Meier et al. 2006; Peterson and Cummings 2006). A standard method of an acid-catalysed trans esterification reaction was used to form fatty acid methyl esters (FAME) by heating the lipid fractions (for a recommended time) with an excess of anhydrous methanol in the presence of an acidic catalyst. The reaction is referred to as "trans-esterification" in general and "trans-methylation" or simply "methylation".

The methylating reagent (15% acetyl chloride in 100ml anhydrous methanol) was prepared freshly by adding acetyl chloride very slowly to cold and dry methanol. 4ml of methylating reagent was transferred to the different lipid fractions (CPG, CE and TG bands) containing methylating tubes and flushed thoroughly with OFN. Immediately after flushing, the methylating tubes were closed tightly and kept for heating in an oven at 70^{o}C for 3 hours. The tubes were removed from the oven at 60, and 120 minutes respectively, vortexed and checked for leakage (indicated by reduced volume than original). If necessary, then made up the volume by adding more methylating reagent, re-flushed and vortexed the sample before putting them back in the oven.

2.2.6.2 Extraction of fatty acids methyl esters

After 3 hours, the methylating tubes were removed from the oven and cooled in dimmed light, at room temperature. 4ml of sodium chloride solution (5% w/v) and 2ml of petroleum ether + BHT (0.01% w/v) was added to each methylating tube (reffered as tube 1). The methylating tubes were closed tightly and shaken well to ensure complete transfer of FAMEs into petroleum ether. Methylating tubes were allowed to settle till two phases formed. 3-5 drops of methanol were added to each tube to break emulsions resulted from vigorously shaking of tubes. The upper FAME containing petrol layer from each methylating tube was transferred to another tube containing 2 ml of (2% w/v) potassium bicarbonate (reffered as tube 2) to neutralize any acid if transferred. 1ml of petroleum ether was again added to the original methylating tube (tube 1), capped and shook vigorously. 2-3 drops of methanol were added again to break the emulsion. The upper FAMEs containing layer of petroleum ether was transferred to the same tube that contained potassium bicarbonate (tube 2).

This entire step is repeated again. In total, FAMEs were extracted with 4ml of petroleum ether (2+1+1ml, 3 times addition).

The tube 2 (FAMEs containing layer of petroleum ether + potassium bicarbonate) was vortexed and two layers formed. The upper FAMEs containing layer of petrol was transferred to a test tube (reffered as tube 3) containing 100-200gm of anhydrous granular sodium sulphate, to ensure removal of any residual water. From tube 3, the FAMEs solution was transferred to a properly labelled 3ml glass vial. Petroleum ether was evaporated under a stream of OFN at 37^{o}C and the sample was taken in 1ml of heptane + BHT (0.01% w/v). The resulting FAMEs were flushed with OFN and stored at -20^{o}C until analysed by gas liquid chromatography.

2.2.7 Analysis of fatty acid methyl esters by gas-liquid chromatography

After extraction of methyl esters, FAMEs were separated by a capillary gas chromatograph (HRGC MEGA 2 series, Fisons Instruments, Italy) fitted with a BPX-70 capillary column (60m × 0.32mm ID, 0.25µm film, SGE Europe Ltd, UK), split injector, and FID. Operating conditions were; hydrogen to column 110Kpa, hydrogen to detector 50Kpa and air to column 70 Kpa. Hydrogen was used as a carrier gas at 2 ml/min and the injector, oven and detector temperatures were 250, 230 and 280^{o}C respectively**.** The oven temperature was initially at 135^{o}C, held for one minute and then rose at the rate of 2^{o}C/minute. Once reached 180^{o}C, it was sustained here again for 1 minute, followed by a further increase at a rate of 0.7^{o}C/minute. After attaining 190^{o}C, it was maintained for 2 minutes, led to further increase to 230^{o}C at the rate of 15^{o}C/minute and maintained here for 2 minutes. The total run time per sample was 42 minutes. Specifications for instrumentation of gas chromatography are enlisted in **Appendix 1** (**Figure 2.6, 2.7**).

Figure 2.6. GLC system used for the analysis of fatty acids methyl esters (picture taken at LNRC lab).

10µl of FAME sample solution (CPG, CE and TG) was prepared and injected. The eluting FAMEs were detected by FID and peak areas were calculated with chromatography data system (Scientific Software Inc., San Ramon, CA). Fatty acids were identified by the comparison of retention times with authentic standards (FAME standard mixture from Supelco® 37 component FAME Mix. U47885-U, Sigma-Aldrich, Dorset, UK and GC-MS authenticated FAMEs) prepared from lipid extracts of olive oils (contain alpha-linolenic acid, gamma-linolenic and stearidonic acids). Peak areas were quantified by a computer chromatography data system (EZChrom Chromatography Data System, Scientific Software Inc., San Ramon, CA), (Ghebremeskel et al. 2000; Min et al. 2004), **(Appendices 3, 7, 8**). The area of each peak was expressed as the percentage of total identified peaks.

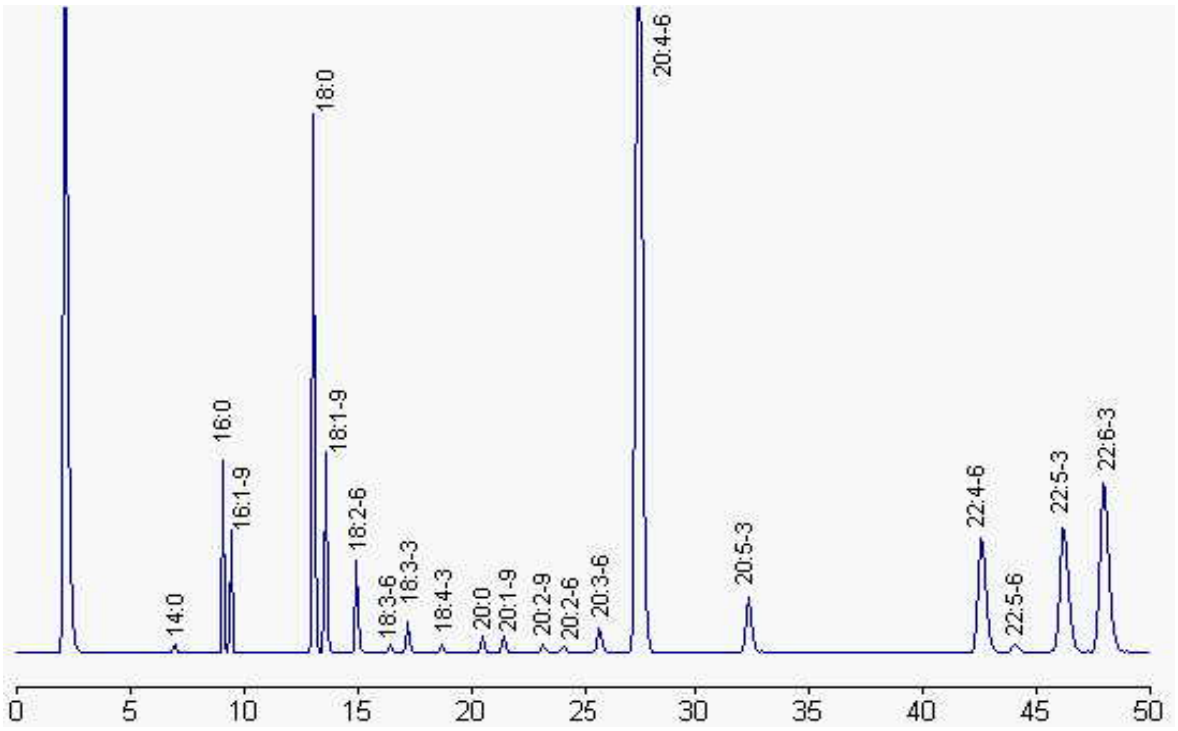

Figure 2.7. A chromatogram of fatty acid methyl esters (adopted from http://lipidlibrary.aocs.org/Analysis/).

2.3 Analysis of plasma alpha-tocopherol, retinol and beta-carotene by high-performance liquid chromatography (HPLC).

2.3.1 Extraction of plasma α-tocopherol, retinol and β-carotene

A volume of 100µl of plasma sample was transferred into a 3ml vial, deproteinized with 500µl of absolute ethanol (with BHT 0.01% w/v) by Vortex. After that, 1ml of hexane (with BHT 0.01% w/v) was added, vortexed thoroughly again for 5 minutes and centrifuged at 3000rpm for 8 minutes. The supernatant was transferred to another well-labeled 3ml glass vial. The sample was extracted again with 1ml hexane as described previously to ensure complete transfer of vitamins into hexane. The whole extract was evaporated under a stream of OFN (at 37^{o}C) and took up into 100µl of a mixture of methanol+ dichloromethane (80:20, with BHT 0.01% w/v). A volume of 25µl of extracted sample was injected through the injector into the column. As mentioned earlier, all extraction procedures were carried out in dim light. The chemicals used and their sources are enlisted in detail in **Appendix 2**.

2.3.2 Separation and identification

Separation and identification of plasma α-tocopherol, retinol and β-carotene were carried out by Agilent 1100 series HPLC (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). The HPLC was connected to an auto sampler, diode-array detector and fitted with a reverse phase column (C18 reverse phase, 5micron, 250 × 4.6nm, Thermo Scientific). Methanol (98% HPLC grade mobile phase) was used as a mobile phase at a flow rate of 1ml/min. The column temperature was maintained at 20^{o}C. α-tocopherol, retinol and β-carotene were detected at their maximum absorption wavelengths of 292, 325 and 453nm respectively. According to the authentic standards, the retention times for α-tocopherol, retinol and β-carotene were 6.4, 4 and 13.8 respectively. The total run-time per sample was 15 minutes. Pure standards of the vitamins were used for authentication (**Appendix 4**). Specifications for instrumentation of HPLC are enlisted in **Appendix 1** (**Figure 2.8, 2.9**).

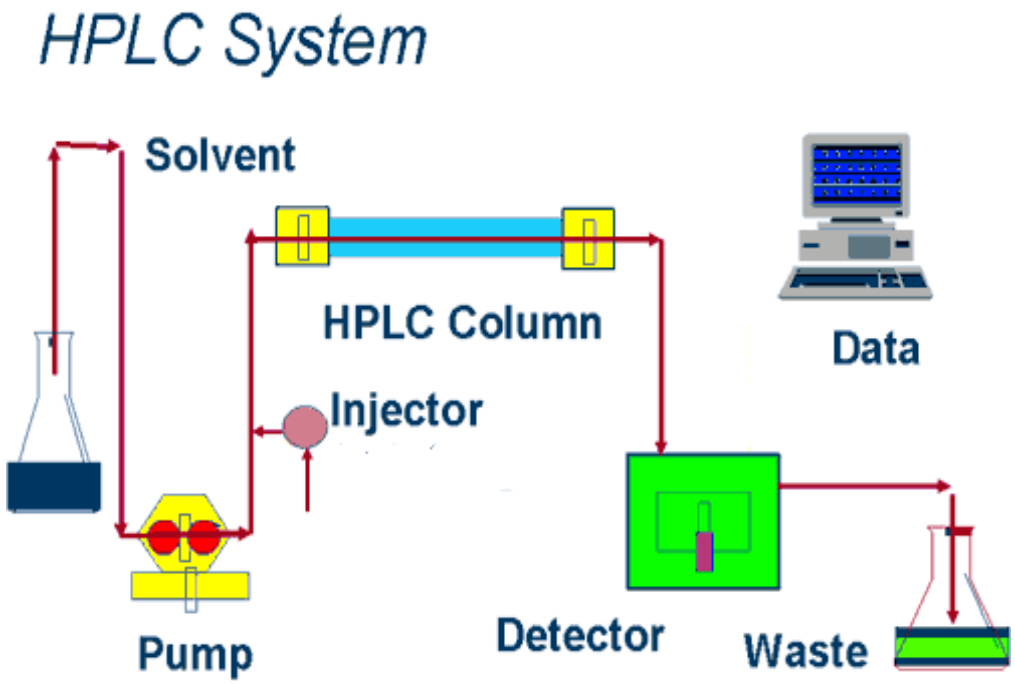

Figure 2.8. A diagramatic representation of HPLC system (adopted from http://web.nmsu.edu/~kburke/Instrumentation/Waters_HPLCSystem.gif).

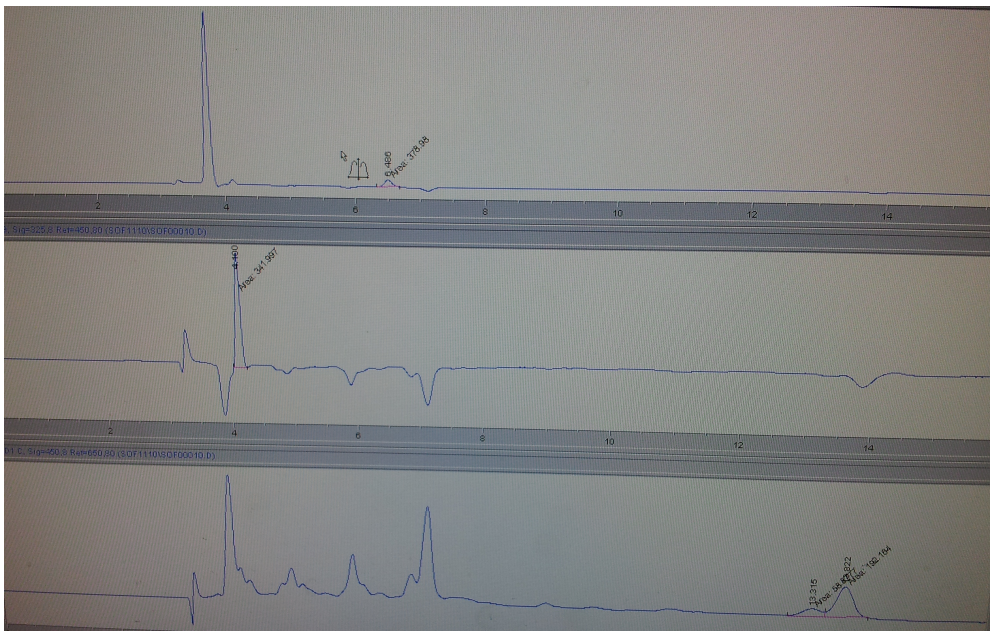

Figure 2.9. A chromatogram showing retention times of retinol, α-tocopherol, and β-carotene, respectively (picture taken at LNRC lab).

2.3.3 Quantification

Standard stock solutions of α-tocopherol (30mg/dl), retinol (600µg/dl) and β-carotene (120µg/dl) were prepared by dissolving the authentic standards into a mixture of methanol and dichloromethane (with BHT, 0.01% w/v, HPLC grade). Working standards for α-tocopherol, retinol and β-carotene were prepared from the stock solutions by serial dilution and used to plot the linear regression lines of concentration versus peak areas. The correlation coefficients (r) of the regression lines were α-tocopherol (r =0.999), retinol (r=0.999) and β-carotene (r=0.999). The concentrations of the vitamins in the samples were calculated by using their respective standard curves (regression line equation obtained from the serial dilution of standard stock solution). Agilent ChemStation (computer software, Agilent Technologies, Germany) was used to compute the peak areas and concentrations of the vitamins. Results are expressed in mg/dl for α-tocopherol while µg/dl for both retinol and β-carotene (**Figure 2.10, 2.11 and 2.12**).

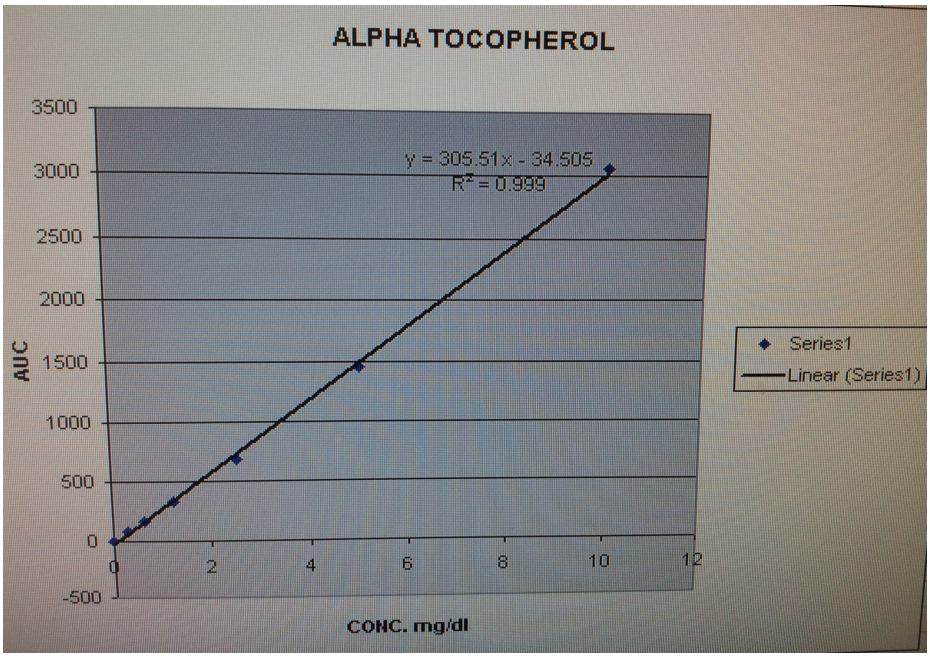

Figure 2.10. Standard curve of α-tocopherol with equation of regression line and correlation coefficient (picture taken at LNRC lab).

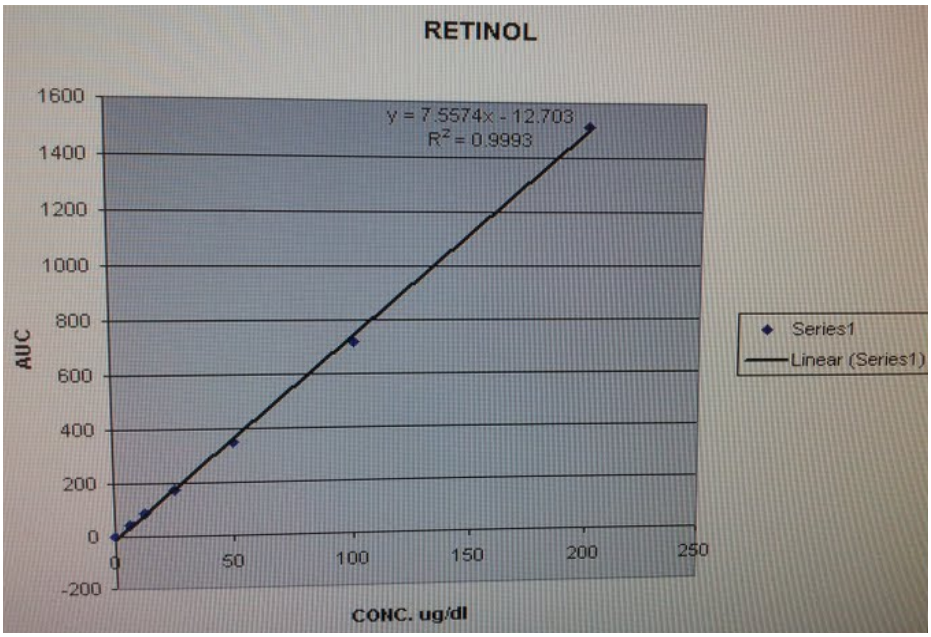

Figure 2.11. Standard curve of retinol with equation of regression line and correlation coefficient (picture taken at LNRC lab).

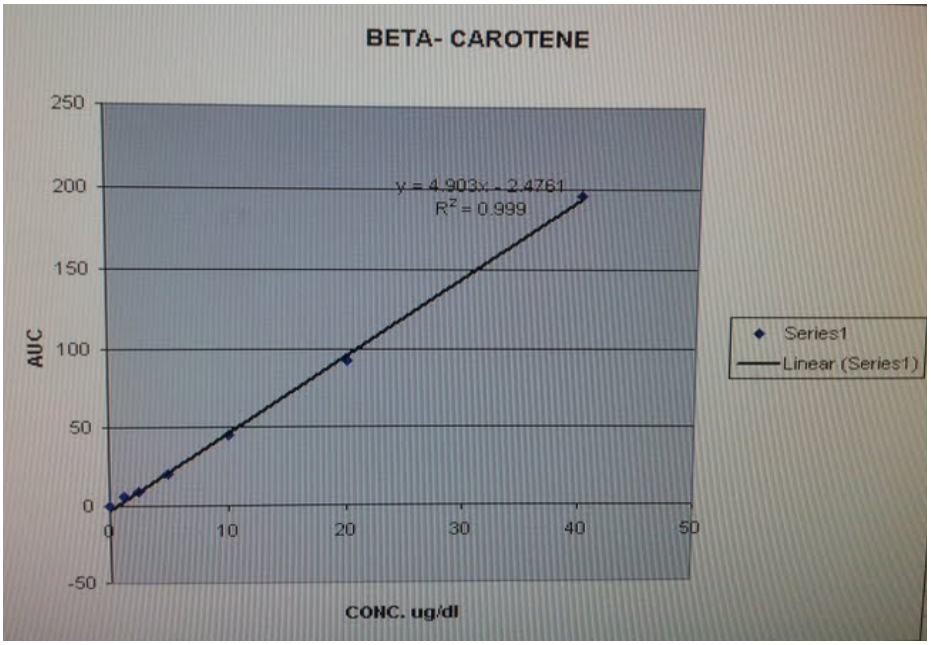

Figure 2.12. Standard curve of β-carotene with equation of regression line and correlation coefficient (picture taken at LNRC lab).

2.4 Data analysis

The data are expressed as mean ± standard deviation (SD), median (range) and n (%), as appropriate. The assumptions of outliers, normality of distribution (Shapiro-Wilk test) and homogeneity of variance (Levene test) for all the data sets were assessed for each statistical test. Depending on the normality of data distribution, parametric (normal distribution) or non-parametric (non-normal distribution) statistical tests were used. The details of each statistical test used to assess significant differences between the groups are discussed in the relevant chapters. Statistical significance was assumed when "*P"* value < 0.05, unless otherwise stated. The statistical analyses were carried out with IBM SPSS Statistics version 24 (IBM Corporation, USA).

CHAPTER 3

3.1 Introduction

Insulin resistance together with perturbations in general lipid metabolism in GDM may affect EFAs metabolism and may ultimately alter placental transfer and foetal LCPUFAs accretion (Clark et al. 1997; Brenner et al. 2000). Other studies (Holman et al. 1983; Poisson and Cunnane 1991) also showed that diabetes impairs the synthesis of long chain ω6 and ω3 PUFAs. Furthermore, the women or/and their offspring having type I, II (Tilvis and Meittinen 1985; Lakin et al. 1998; Ghebremeskel et al. 2004; Min et al. 2005[b]), or GDM have reduced levels of membrane AA and DHA (Wijendran et al. 2000; Min et al. 2004; Min et al. 2005[a]; Pagan et al. 2013).

DHA is a long-chain polyunsaturated n-3 fatty acid with bioactive properties, and it is a key nutrient for optimal foetal neurovisual development. Furthermore, it is thought to be an effective anti-adiposity agent (Ruzickova et al. 2004). A growing body of evidence suggested that there is an inverse association between n-3 LCPUFAs and insulin resistance (IR), (Soria et al. 2002; Pighin et al. 2003). n-3 supplementation has also increased insulin-stimulated glucose transport in supplemented animals (Peyron-Caso et al. 2002). n-3 LCPUFAs are proposed to decrease the risk of insulin resistance by multiple means, few of which seem to be distinctively affected by n-3 fatty acids. DHA and EPA are favorably incorporated into cell membranes, hence increasing membrane fluidity, up-regulating insulin receptors, decrease insulin resistance and favorably modify an individual's glycemic response, an effect that could possibly delay or avert the onset of type II diabetes mellitus (Das 2005).

Since diabetes impairs the synthesis of long-chain ω3 PUFAs, it is plausible that the increased dietary intake of ω3-LCPUFAs during pregnancy associated with GDM may rectify the required maternal and foetal LCPUFAs levels. A number of studies have investigated the beneficial effects of ω-3 fatty acid supplementation in the enhancement of maternal and neonatal fatty acids status in normal pregnancy (Dunstan et al. 2004; Escolano-Margarit et al. 2013). To the best of our knowledge, no clinical study has been conducted to evaluate the effect of dietary ω3 supplementation on LCPUFAs status (particularly DHA) of GDM mothers and their neonates. Hence, we carried out a double-blind, randomized, placebo-controlled trial to investigate if ω3-

enriched fish oil supplementation enhances the plasma fatty acids status, particularly DHA, in women with GDM.

3.2 Subjects and methods

The details of subjects and methods are described in **Chapter 2.**

3.3 Statistical analysis

The data are expressed as mean ± standard deviation (SD), n (%) and median (range) as appropriate. Statistical significance was assumed at $P < 0.05$ unless otherwise stated. Independent samples t-test (for equal variances) or Welch t-test (for unequal variances) was run to explore the significant differences in the plasma fatty acid levels between healthy pregnant and GDM women, at baseline. One-way analysis of variance (ANOVA) was used to compare the differences in;

- Demographic and obstetric data of healthy pregnant and GDM women (placebo and active-treatment groups).
- Plasma fatty acids composition between healthy pregnant and GDM women (placebo and active-treatment groups), at delivery.

A pairwise comparison was performed using Tukey's honest significant difference (HSD, for equal variance) and Games-Howell (for unequal variance) tests for each dependent variable separately when the F-ratio was significant ($P < 0.05$). All statistical analyses were carried out with IBM SPSS Statistics version 24 (IBM Corporation, USA).

3.4 Results

3.4.1 Clinical and demographic characteristics of the participants

170 women in total, of which 142 with GDM and 28 with healthy pregnancy were randomized to either placebo or ω-3 enriched active treatment group (**Figure 3.1**). 117 women with GDM (active group, n = 61; placebo group, n = 56) and 23 healthy pregnant women (active group, n = 9; placebo group, n = 14) completed the trial. The detailed characteristics of the participants are given in **Table 3.1**.

Although the differences were not significant, the BMI were higher in GDM women (active and placebo groups) as compared to healthy pregnant women. GDM women (active-treatment and placebo groups) also had higher blood glucose concentrations as compared to both healthy pregnant active- and placebo-groups, at 0 ($P < 0.0001$) and 120min ($P < 0.0001$) after the oral glucose tolerance test (OGTT). The majority of the subjects were of Asian (55.9%) and African/Afro-Caribbean (28.2%) origin. 23.6% of the GDM women from active-group and 28.6% from placebo-group had gestational diabetes in their previous pregnancy. Over 60% of GDM women were treated with oral hypoglycemic agents (active-group 33.3%; placebo-group 32.9%), insulin (active-group 25.0%; Placebo-group 20.0%) or oral hypoglycaemic agents + insulin (active-group 2.8%; Placebo-group 8.6%).

There was a wide variation in the duration of supplementation. This was because of a number of women being diagnosed with GDM either early or late stage of pregnancy. 6 GDM women (4 in active- and 2 in placebo-group) and 1 healthy pregnant woman (placebo) claimed to have occasionally taken omega-3 supplement purchased from health shops during the course of the study. However, their DHA and EPA levels were not different from the mean values of their respective groups. Compliance was monitored by regular home visits by the research midwives and telephone contact (including out of office hour), and by counting unused capsules which the women were asked to bring with them before dispensing the next batch of supplements.

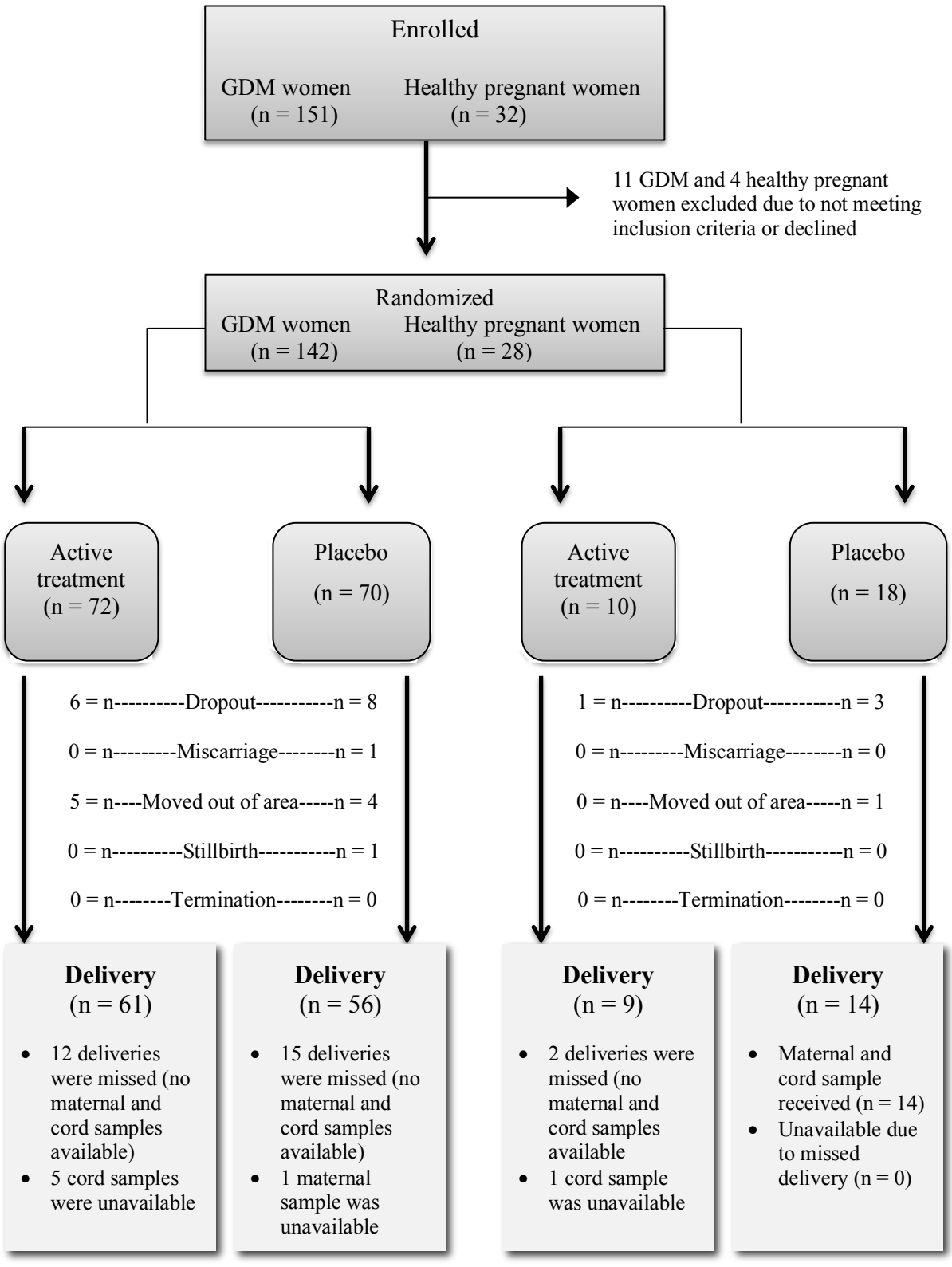

Figure 3.1. Flowchart of clinical trial showing 170 women (142 with GDM and 28 with normal pregnancy), randomized to either dietary omega-3 supplementation or placebo.

Table 3.1. Demographic and obstetric variables of the participants.

	GDM women		Healthy pregnant women	
	Active-group	Placebo-group	Active-group	Placebo-group
Number of participants (*n*)[a]	72	70	10	18
Gestation at recruitment (weeks)[b]	28.0 (17.0-34.0)	27.5 (17.0-32.0)	27.0 (23.0-31.0)	27.0 (17.0-29.0)
Age (years)[b]	30.0 (20.0-44.0)	32.0 (19.0-43.0)	31.0 (21.0-40.0)	31.0 (17.0-39.0)
Height (m)[c]	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Pre-pregnancy weight (kg)[c]	74.1±15.8	72.9±15.7	69.5±17.5	68.1±12.3
Pre-pregnancy BMI (kg/m^2)[c]	29.0±5.5	29.3±5.7	27.2±4.8	25.7±4.5
≤ 25.0 (*n*)[d]	19 (26.4)	25 (35.7)	04 (40.0)	10 (55.6)
25.1-30.0 (*n*)[d]	27 (37.5)	20 (28.6)	03 (30.0)	06 (33.3)
> 30.0 (*n*)[d]	26 (36.1)	25 (35.7)	03 (30.0)	02 (11.1)
Racial Origin (*n*)[d, e]				
Asian	44 (61.1)	42 (60.0)	4 (40.0)	5 (27.8)
African/Afro-Caribbean	18 (25.0)	18 (25.7)	5 (50.0)	7 (38.9)
Caucasian	6 (8.3)	6 (8.6)	0 (0.0)	6 (33.3)
Others	4 (5.6)	4 (5.7)	1 (10.0)	0 (0.0)
Smoker (*n*)[d]	2 (2.8)	0 (0.0)	0 (0.0)	2 (11.1)
Planned pregnancy (*n*)[d]	43 (59.7)	35 (50.0)	3 (30.0)	12 (66.7)
Parity (*n*)[d]				
0	33 (45.8)	22 (31.4)	3 (30.0)	10 (55.6)
1-2	24 (33.3)	34 (48.6)	5 (50.0)	6 (33.3)
≥ 3	15 (20.8)	14 (20.0)	2 (20.0)	2 (11.1)
GDM in previous pregnancy (*n*)[d]	17 (23.6)	20 (28.6)	0 (0.0)	0 (0.0)
Folic acid use (*n*)[d]	41 (56.9)	40 (57.1)	7 (70.0)	9 (50.0)
Family history of diabetes (*n*)[d, f]	41 (56.9)	38 (54.3)	0 (0.0)	8 (44.4)
Glucose tolerance test				
Gestation (weeks)[b]	19.5 (12.0-31.0)	20.0 (12.0-31.0)	28.0 (27.0-31.0)	28.0 (17.0-32.0)
Glucose at 0 min (mmol)[c]	5.6±1.6^{***+++}	5.5±1.5^{***+++}	4.2±0.5	4.2±0.4
Glucose at 120 min (mmol)[c]	9.4±2.1^{***+++}	9.2±1.6^{***+++}	5.4±1.1	5.3±1.0
Diabetes treatment (*n*)[d]				
Diet only	28 (38.9)	27 (38.6)	0 (0.0)	0 (0.0)
Oral hypoglycemic agents	24 (33.3)	23 (32.9)	0 (0.0)	0 (0.0)
Insulin	18 (25.0)	14 (20.0)	0 (0.0)	0 (0.0)
Oral hypoglycemic + insulin	2 (2.8)	6 (8.6)	0 (0.0)	0 (0.0)

[a] This number included all the subjects who enrolled at the beginning of this trial regardless of the dropout later.
[b] Data are expressed as median (mininum - maximum)
[c] Data are expressed as mean ± SD
[d] Data are expressed as number of subjects (%)
[e] We classified individuals according to the UK Home Office's classification for an individual's ethnicity which is based on person's self-defination (**African/Afro-Caribbean**: Afro-Caribbean, Black African, Black British, Caribbean; **Asian**: Bangladesh, Bengali, Indian, Pakistani, Sri Lankan; **Caucasian**: English, European, Irish, Polish; **Others**: Arab, Filipino, Latin American, North African, mixed race).
[f] Information was not available from five women with GDM (1 active-group, 4 placebo-group).
Healthy active versus GDM active group and GDM placebo group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$
Healthy placebo versus GDM active group and GDM placebo group: $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$

3.4.2 Maternal plasma fatty acid composition at baseline

This result reports plasma choline phosphoglycerides, cholesterol esters, and triglycerides fatty acids of the GDM (n = 142) and healthy pregnant women (n = 28) at diagnosis, matched for the gestational period.

3.4.2.1 Plasma choline phosphoglycerides (CPG)

Fatty acid composition of plasma CPG at baseline is presented in **Table 3.2**. Compared with healthy pregnant women, the GDM women had lower levels of 22:5n-3 (docosapentaenoic acid; $P < 0.01$). The levels of other fatty acids were comparable between the healthy pregnant and GDM women ($P > 0.05$).

3.4.2.2 Plasma cholesterol esters (CE)

Fatty acid composition of plasma CE at baseline is presented in **Table 3.3**. Compared with healthy pregnant women, the GDM women had lower levels of 18:1n-9 (oleic acid) and Σ monounsaturates ($P < 0.05$). The levels of other fatty acids were comparable between the healthy pregnant and GDM women ($P > 0.05$).

3.4.2.3 Plasma triglycerides (TG)

Fatty acid composition of plasma TG at baseline is presented in **Table 3.4**. Compared with healthy pregnant women, the GDM women had lower levels of 14:0 (myristic acid; $P < 0.05$). The levels of other fatty acids were comparable between the healthy pregnant and GDM women ($P > 0.05$).

Table 3.2. Mean (±SD) percent fatty acid composition of plasma choline phosphoglycerides of healthy pregnant and GDM women at baseline.

Fatty acids	**Healthy pregnant women** (n = 28)	**GDM women** (n = 142)
14:0	0.2 ± 0.1	0.2 ± 0.1
16:0	29.7 ± 3.5	30.2 ± 3.3
18:0	10.5 ± 1.3	10.1 ± 1.3
20:0	0.04 ± 0.01	0.04 ± 0.02
22:0	0.1 ± 0.1	0.1 ± 0.1
24:0	0.1 ± 0.03	0.1 ± 0.02
Σ Saturates	41.1 ± 2.7	41.4 ± 2.8
16:1n-7	0.4 ± 0.3	0.4 ± 0.2
18:1n-7	1.4 ± 0.3	1.4 ± 0.4
18:1n-9	9.8 ± 2.3	9.5 ± 2.7
24:1n-9	0.04 ± 0.02	0.05 ± 0.03
Σ Monoenes	12.0 ± 2.5	11.5 ± 3.1
18:2n-6	24.2 ± 3.1	24.8 ± 3.2
18:3n-6	0.1 ± 0.04	0.1 ± 0.1
20:2n-6	0.5 ± 0.1	0.4 ± 0.1
20:3n-6	3.9 ± 1.0	3.6 ± 0.9
20:4n-6	10.0 ± 1.9	10.6 ± 2.1
22:4n-6	0.3 ± 0.1	0.3 ± 0.2
22:5n-6	0.4 ± 0.2	0.4 ± 0.3
Σ n-6	39.3 ± 3.7	40.3 ± 3.4
18:3n-3	0.3 ± 0.1	0.3 ± 0.1
20:5n-3	0.8 ± 0.5	0.7 ± 0.6
22:5n-3	0.7 ± 0.2**	0.5 ± 0.2
22:6n-3	4.9 ± 1.7	4.4 ± 1.1
Σ n-3	6.8 ± 2.2	6.0 ± 1.7

Healthy pregnant women versus GDM women: $^{*}P < 0.05$, $^{**}P < 0.01$

Table 3.3. Mean (±SD) percent fatty acid composition of plasma cholesterol esters of healthy pregnant and GDM women at baseline.

Fatty acids	**Healthy pregnant women** (n = 28)	**GDM women** (n = 142)
14:0	0.3 ± 0.2	0.3 ± 0.2
16:0	9.5 ± 1.7	9.5 ± 1.8
18:0	0.5 ± 0.1	0.5 ± 0.1
20:0	0.05 ± 0.02	0.04 ± 0.02
22:0	0.03 ± 0.03	0.03 ± 0.02
24:0	tr	tr
Σ Saturates	11.6 ± 2.6	12.1 ± 3.1
16:1n-7	1.9 ± 1.3	1.6 ± 1.0
18:1n-7	1.1 ± 0.2	1.0 ± 0.2
18:1n-9	17.4 ± 3.6*	15.8 ± 2.8
24:1n-9	0.8 ± 0.5	0.7 ± 0.4
Σ Monoenes	21.2 ± 4.3*	19.1 ± 3.4
18:2n-6	54.9 ± 5.1	55.5 ± 5.4
18:3n-6	0.6 ± 0.3	0.5 ± 0.2
20:2n-6	0.1 ± 0.02	0.1 ± 0.02
20:3n-6	0.9 ± 0.2	0.9 ± 0.2
20:4n-6	8.0 ± 1.7	8.6 ± 2.0
22:4n-6	0.1 ± 0.04	0.1 ± 0.1
22:5n-6	0.1 ± 0.1	0.1 ± 0.1
Σ n-6	64.7 ± 5.1	65.8 ± 5.4
18:3n-3	0.7 ± 0.3	0.7 ± 0.3
20:5n-3	0.8 ± 0.5	0.7 ± 0.6
22:5n-3	0.1 ± 0.02	0.1 ± 0.1
22:6n-3	0.9 ± 0.4	0.9 ± 0.3
Σ n-3	2.6 ± 0.9	2.4 ± 0.9

tr - trace
Healthy pregnant women versus GDM women: $^{*}P < 0.05$

Table 3.4. Mean (±SD) percent fatty acid composition of plasma triglycerides of healthy pregnant and GDM women at baseline.

Fatty acids	**Healthy pregnant women** (n = 28)	**GDM women** (n = 142)
14:0	1.6 ± 0.8*	1.2 ± 0.7
16:0	28.4 ± 4.8	26.6 ± 4.0
18:0	3.0 ± 0.5	3.0 ± 0.6
20:0	0.1 ± 0.02	0.1 ± 0.04
22:0	0.1 ± 0.1	0.04 ± 0.03
24:0	tr	tr
Σ Saturates	34.2 ± 5.5	32.1 ± 4.7
16:1n-7	2.4 ± 1.4	1.9 ± 0.8
18:1n-7	2.0 ± 0.4	2.1 ± 0.4
18:1n-9	33.3 ± 5.5	33.5 ± 5.0
24:1n-9	0.04 ± 0.03	0.03 ± 0.02
Σ Monoenes	38.3 ± 5.4	38.0 ± 5.2
18:2n-6	21.1 ± 6.2	23.3 ± 5.7
18:3n-6	0.3 ± 0.2	0.3 ± 0.1
20:2n-6	0.3 ± 0.1	0.3 ± 0.1
20:3n-6	0.4 ± 0.1	0.4 ± 0.1
20:4n-6	1.5 ± 0.6	1.4 ± 0.4
22:4n-6	0.2 ± 0.1	0.2 ± 0.1
22:5n-6	0.3 ± 0.2	0.3 ± 0.1
Σ n-6	24.1 ± 6.7	26.2 ± 6.1
18:3n-3	1.1 ± 0.4	1.3 ± 0.6
20:5n-3	0.2 ± 0.1	0.2 ± 0.2
22:5n-3	0.2 ± 0.1	0.2 ± 0.1
22:6n-3	0.9 ± 0.4	0.9 ± 0.6
Σ n-3	2.6 ± 0.8	2.8 ± 1.1

tr – trace
Healthy pregnant women versus GDM women: $^{*}P < 0.05$

3.4.3 Maternal plasma fatty acid composition at delivery

3.4.3.1 Plasma choline phosphoglycerides (CPG)

Fatty acid composition of plasma CPG is given in **Table 3.5**. The healthy placebo-group had higher levels of 16:1n-7 (palmitoleic acid; $P < 0.05$), 18:1n-9 (oleic acid; $P < 0.05$), Σ monoenes ($P < 0.05$) and 20:3n-6 (dihomo-γ-linolenic acid; $P < 0.01$) compared with healthy active-treatment group. In comparison with healthy placebo, GDM placebo-group had lower levels of 16:1n-7 (palmitoleic acid; $P < 0.01$), 18:1n-7 (vaccenic acid; $P < 0.05$), 18:1n-9 (oleic acid; $P < 0.05$), Σ monoenes ($P < 0.01$) and 20:3n-6 (dihomo-γ-linolenic acid; $P < 0.05$) but high Σ n-6 fatty acids ($P < 0.05$). GDM active-treatment group in comparison with healthy placebo-group, had lower levels of 16:1n-7 (palmitoleic acid; $P < 0.001$), 18:1n-7 (vaccenic acid; $P < 0.05$), 18:1n-9 (oleic acid; $P < 0.01$), Σ monoenes ($P < 0.001$) and 20:3n-6 (dihomo-γ-linolenic acid; $P < 0.05$) but high 18:2n-6 (linoleic acid; $P < 0.01$) and Σ n-6 fatty acids ($P < 0.01$). DHA (22:6n-3; $P < 0.05$) was increased in GDM active-treatment group compared with GDM placebo. Although not significantly, but DHA (22:6n-3) was high in healthy active-treatment group as compared to the healthy-placebo.

3.4.3.2 Plasma cholesterol esters (CE)

Table 3.6 shows plasma CE fatty acid levels of all four groups. 16:1n-7 (palmitoleic acid; $P < 0.01$) was elevated in healthy placebo-group than in healthy active-treatment group. GDM placebo compared to healthy placebo-group had lower levels of 16:1n-7 (palmitoleic acid; $P < 0.001$), 18:1n-9 (oleic acid; $P < 0.001$) and Σ monoenes ($P < 0.05$) but higher Σ n-6 fatty acids ($P < 0.001$). In comparison with healthy placebo-group, GDM active-treatment group also had lower levels of 16:1n-7 (palmitoleic acid; $P < 0.001$), 18:1n-9 (oleic acid; $P < 0.001$) and Σ monoenes ($P < 0.05$) but higher Σ n-6 fatty acids ($P < 0.001$). DHA (22:6n-3) was elevated in both GDM active ($P < 0.01$) and healthy active-treatment (not significantly) groups than their placebo-treated counterparts (GDM-placebo and healthy-placebo).

3.4.3.3 Plasma triglycerides (TG)

Mean fatty acids of the plasma TG is presented in **Table 3.7**. The healthy placebo-group compared with the healthy active-treatment group had reduced 18:2n-6 (linoleic acid; $P < 0.01$), Σ n-6 ($P < 0.01$) and increased 16:1n-7 (palmitoleic acid; $P <$

0.01) and Σ monoenes ($P < 0.05$). GDM placebo compared with healthy placebo-group had lower 16:1n-7 (palmitoleic acid; $P < 0.001$) but higher 18:2n-6 (linoleic acid; $P < 0.01$) and Σ n-6 fatty acids ($P < 0.01$). GDM active-treatment group compared with healthy-placebo had lower 16:1n-7 (palmitoleic acid; $P < 0.001$) and Σ monoenes ($P < 0.01$) but higher 18:2n-6 (linoleic acid; $P < 0.001$) and Σ n-6 fatty acids ($P < 0.01$). DHA (22:6n-3; $P < 0.05$) was elevated in GDM active-treatment group compared with GDM placebo. Although it did not reach the level of a statistical significance, the mean percentage of DHA (22:6n-3) was also higher in the healthy active-treatment group as compared to healthy placebo.

Table 3.5. Mean (±SD) percent fatty acid composition of maternal plasma choline phosphoglycerides at delivery.

Fatty acids	Healthy pregnant women (n = 21)		GDM women (n = 89)	
	Placebo group (n = 14)	Active group (n = 7)	Placebo group (n = 40)	Active group (n = 49)
14:0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
16:0	31.7 ± 3.7	31.8 ± 1.3	32.7 ± 2.4	32.2 ± 2.2
18:0	9.1 ± 2.4	10.1 ± 1.9	8.9 ± 1.2	9.0 ± 1.1
20:0	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
22:0	0.2 ± 0.1	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.03
24:0	tr	tr	tr	tr
Σ Saturates	41.9 ± 1.8	42.9 ± 2.3	42.7 ± 2.3	42.3 ± 2.0
16:1n-7	$1.0 \pm 0.3^{*}$	0.6 ± 0.4	$0.7 \pm 0.3^{++}$	$0.6 \pm 0.3^{\times\times\times}$
18:1n-7	1.7 ± 0.4	1.5 ± 0.3	$1.4 \pm 0.2^{+}$	$1.4 \pm 0.3^{\times}$
18:1n-9	$14.0 \pm 1.8^{*}$	11.0 ± 1.2	$11.9 \pm 2.5^{+}$	$11.6 \pm 2.1^{\times\times}$
24:1n-9	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02
Σ Monoenes	$16.9 \pm 2.0^{*}$	13.4 ± 1.4	$14.2 \pm 2.8^{++}$	$13.8 \pm 2.4^{\times\times\times}$
18:2n-6	20.1 ± 3.7	21.8 ± 4.1	22.7 ± 3.7	$23.8 \pm 3.1^{\times\times}$
18:3n-6	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.04
20:2n-6	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
20:3n-6	$3.9 \pm 1.2^{**}$	2.7 ± 0.7	$3.2 \pm 0.8^{+}$	$3.2 \pm 0.8^{\times}$
20:4n-6	9.5 ± 3.0	10.4 ± 3.0	10.0 ± 2.3	9.2 ± 1.7
22:4n-6	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
22:5n-6	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
Σ n-6	34.8 ± 1.8	35.8 ± 2.6	$37.0 \pm 4.1^{+}$	$37.2 \pm 3.0^{\times\times}$
18:3n-3	0.5 ± 0.2	0.3 ± 0.2	0.4 ± 0.1	0.4 ± 0.1
20:5n-3	0.7 ± 0.5	1.5 ± 1.4	0.6 ± 0.5	0.7 ± 0.4
22:5n-3	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.1
22:6n-3	3.8 ± 1.5	4.8 ± 1.3	3.7 ± 1.1	$4.4 \pm 1.1^{\neq}$
Σ n-3	5.6 ± 1.9	7.3 ± 2.8	5.3 ± 1.6	6.0 ± 1.4

tr - trace
Healthy placebo versus healthy active group: $^{*}P < 0.05$, $^{**}P < 0.01$
Healthy placebo versus GDM placebo group: $^{+}P < 0.05$, $^{++}P < 0.01$
Healthy placebo versus GDM active group: $^{\times}P < 0.05$, $^{\times\times}P < 0.01$, $^{\times\times\times}P < 0.001$
GDM Placebo versus GDM active group: $^{\neq}P < 0.05$

Table 3.6. Mean (±SD) percent fatty acid composition of maternal plasma cholesterol esters at delivery.

Fatty acids	Healthy pregnant women (n = 21)		GDM women (n = 89)	
	Placebo group (n = 14)	Active group (n = 7)	Placebo group (n = 40)	Active group (n = 49)
14:0	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
16:0	11.0 ± 1.8	10.3 ± 2.4	9.6 ± 1.6	9.5 ± 1.8
18:0	0.7 ± 0.7	0.9 ± 1.0	0.6 ± 0.9	0.5 ± 0.1
20:0	0.1 ± 0.03	0.04 ± 0.03	0.1 ± 0.1	0.1 ± 0.1
22:0	0.1 ± 0.04	0.04 ± 0.02	0.1 ± 0.1	0.1 ± 0.1
24:0	tr	tr	tr	tr
Σ Saturates	13.5 ± 3.0	12.7 ± 3.3	11.8 ± 2.7	11.5 ± 2.1
16:1n-7	4.5 ± 1.8**	2.2 ± 1.5	1.9 ± 1.1^{+++}	1.7 ± 1.0$^{\times\times\times}$
18:1n-7	1.5 ± 1.0	1.4 ± 0.8	1.0 ± 0.2	1.0 ± 0.2
18:1n-9	22.9 ± 4.6	19.0 ± 5.4	17.1 ± 2.4^{+++}	16.8 ± 3.0$^{\times\times\times}$
24:1n-9	0.2 ± 0.2	0.3 ± 0.3	0.4 ± 0.2	0.4 ± 0.2
Σ Monoenes	29.2 ± 5.8	22.9 ± 6.7	20.4 ± 3.0^{+}	20.0 ± 3.5$^{\times}$
18:2n-6	44.2 ± 10.8	49.8 ± 11.8	54.5 ± 4.8	55.4 ± 4.4
18:3n-6	0.8 ± 0.3	0.5 ± 0.2	0.6 ± 0.3	0.6 ± 0.3
20:2n-6	0.05 ± 0.03	0.04 ± 0.02	0.1 ± 0.1	0.1 ± 0.1
20:3n-6	1.1 ± 0.4	0.8 ± 0.3	0.9 ± 0.2	0.9 ± 0.3
20:4n-6	7.7 ± 2.8	9.1 ± 3.5	8.7 ± 2.3	8.1 ± 2.0
22:4n-6	0.1 ± 0.1	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.1
22:5n-6	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
Σ n-6	54.1 ± 7.6	60.5 ± 9.3	65.0 ± 4.9^{+++}	65.3 ± 4.3$^{\times\times\times}$
18:3n-3	0.9 ± 0.5	0.5 ± 0.3	0.6 ± 0.3	0.6 ± 0.3
20:5n-3	0.6 ± 0.4	1.4 ± 1.3	0.7 ± 0.6	0.7 ± 0.4
22:5n-3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.2
22:6n-3	0.8 ± 0.3	1.2 ± 0.3	0.9 ± 0.3	1.1 ± 0.3$^{\neq\neq}$
Σ n-3	2.5 ± 0.9	3.4 ± 1.7	2.4 ± 0.9	2.8 ± 0.8

tr - trace
Healthy placebo versus healthy active group: $^{*}P < 0.05$, $^{**}P < 0.01$
Healthy placebo versus GDM placebo group: $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$
Healthy placebo versus GDM active group: $^{\times}P < 0.05$, $^{\times\times}P < 0.01$, $^{\times\times\times}P < 0.001$
GDM Placebo versus GDM active group: $^{\neq}P < 0.05$, $^{\neq\neq}P < 0.01$

Table 3.7. Mean (±SD) percent fatty acid composition of maternal plasma triglycerides at delivery.

Fatty acids	**Healthy pregnant women** (n = 21)		**GDM women** (n = 89)	
	Placebo group (n = 14)	**Active group** (n = 7)	**Placebo group** (n = 40)	**Active group** (n = 49)
14:0	0.7 ± 0.3	0.8 ± 0.7	0.6 ± 0.3	0.7 ± 0.3
16:0	29.0 ± 3.1	25.6 ± 3.0	26.8 ± 3.3	27.4 ± 2.5
18:0	2.9 ± 0.9	3.1 ± 1.0	2.6 ± 0.5	2.7 ± 0.6
20:0	0.1 ± 0.04	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.04
22:0	0.04 ± 0.01	0.05 ± 0.04	0.1 ± 0.2	0.05 ± 0.1
24:0	tr	tr	tr	tr
Σ Saturates	33.7 ± 2.7	30.4 ± 3.7	31.0 ± 3.5	31.7 ± 2.6
16:1n-7	$3.1 \pm 1.1^{**}$	1.8 ± 1.0	$1.9 \pm 0.8^{+++}$	$1.8 \pm 0.7^{\times\times\times}$
18:1n-7	2.8 ± 0.7	2.1 ± 0.4	2.3 ± 0.3	2.1 ± 0.4
18:1n-9	38.2 ± 3.4	33.8 ± 2.2	36.7 ± 3.5	34.9 ± 4.3
24:1n-9	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.1
Σ Monoenes	$44.6 \pm 3.4^{*}$	38.1 ± 2.0	41.3 ± 3.8	$39.1 \pm 4.7^{\times\times}$
18:2n-6	$15.0 \pm 2.8^{**}$	22.9 ± 2.2	$21.6 \pm 4.9^{++}$	$22.6 \pm 4.5^{\times\times\times}$
18:3n-6	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
20:2n-6	0.2 ± 0.04	0.3 ± 0.1	0.3 ± 01	0.3 ± 0.1
20:3n-6	0.4 ± 0.3	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
20:4n-6	1.5 ± 0.6	1.9 ± 0.9	1.4 ± 0.4	1.4 ± 0.5
22:4n-6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 01
22:5n-6	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 01	0.2 ± 0.1
Σ n-6	$18.0 \pm 2.8^{**}$	26.3 ± 2.8	$24.3 \pm 5.3^{++}$	$25.2 \pm 4.8^{\times\times}$
18:3n-3	0.8 ± 0.4	0.8 ± 0.4	0.9 ± 0.3	0.9 ± 0.3
20:5n-3	0.2 ± 0.1	0.6 ± 0.6	0.2 ± 0.1	0.2 ± 0.2
22:5n-3	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
22:6n-3	0.9 ± 0.6	1.7 ± 1.0	0.8 ± 0.4	$1.2 \pm 0.8^{\neq}$
Σ n-3	2.5 ± 1.2	3.7 ± 1.9	2.4 ± 0.7	2.8 ± 1.1

tr - trace
Healthy placebo versus healthy active group: $^{*}P < 0.05$, $^{**}P < 0.01$
Healthy placebo versus GDM placebo group: $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$
Healthy placebo versus GDM active group: $^{\times}P < 0.05$, $^{\times\times}P < 0.01$, $^{\times\times\times}P < 0.001$
GDM Placebo versus GDM active group: $^{\neq}P < 0.05$, $^{\neq\neq}P < 0.01$

3.5 Discussion and conclusion

This study shows that the GDM women were heavier (BMI ≥ 29) and of higher parity (parity ≥ 3) as compared to non-diabetic pregnant women. These findings corroborate earlier reports, demonstrating a BMI ≥ 29 kg/m^2 (Ramos and Caughey 2005; Chu et al. 2007; Shah et al. 2011; Khan et al. 2013) and high parity (Al-Rowaily and Abolfotouh 2010; Khan et al. 2013) are important risk factors for gestational diabetes. The GDM women in our study did not show significant difference in age when compared to the healthy pregnant women. However, on further evaluating the demographic data, 40 GDM women were ≥ 35 years old as compared to only 7 healthy pregnant women in this age category, although the sample size of latter group was small. The relationship between parity and diabetes is strongly associated to obesity and age. Women with higher parity tend to be older and obese. However, age is a confounding factor in the association between parity and GDM. Hence, while evaluating parity the control for age cannot be ignored. The strength of this association, on the other hand could diminish when the adjustments for BMI are made. The association between high parity and the risk of developing GDM could be better studied through a hierarchical model (Dode and dos Santos 2009).

A vast majority of GDM women were of Asian (approximately 60%, mainly Bangladeshi) and African/Afro-Caribbean (approximately 25%) origin. Although, Newham Borough (the resident population area) has one of the highest ethnic minority populations among all the districts in the country, but with no particular ethnic group dominating. Also, the subjects were recruited at random. This disproportionate representation of women of Asian and African/Afro-Caribbean origin in the GDM group proposes that they may have higher chances of developing GDM. It has been considered for long that some ethnicities of women are at high risk of developing GDM (Yuen and Wong 2015). For example, Africans and South-Indians women were at higher risk of developing GDM regardless of BMI (Makgoba et al. 2012). Another study has shown that South Asian women were at higher risk (7.1%) of developing GDM than Caucasian women (Savitz et al. 2008). It is plausible that genetic predisposition, lifestyle and dietary habits including less physical activity and a high intake of saturated fat promoting obesity may be partially responsible for the disproportionate prevalence of the GDM among the Asian and Afro-Caribbean/African women in the current study.

In pregnancies complicated by GDM, some studies have shown comparable levels of LCPUFAs particularly DHA and AA (to their controls) in maternal plasma phospholipids (Wijendran et al. 1999) and triglycerides (Min et al. 2005[a]). Even higher levels of DHA and AA have also been reported in maternal plasma phospholipids (Ghebremeskel et al. 1998; Thomas et al. 2004) and neutral lipids (Thomas et al. 2004). In corroboration to the studies mentioned above, this study has also shown that the GDM women compared with their healthy control, have apparently normal DHA and AA levels in plasma CPG, CE and TG lipid fractions. There is no clear explanation for this relatively equivalent DHA and AA status in the three plasma lipid fractions (CPG, CE, TG) of the GDM women. Though, it could be due to higher intake, mobilisation or insulin-induced increased synthesis. Since the blood samples were collected at diagnosis before the start of any medical treatment, insulin therapy or diet could not have been a factor. Raised plasma fatty acids are a common sign of insulin resistance (Unger and Foster 1998; Nelson et al. 2010; Karpe et al. 2011). Thus, it is possible that a persistently high concentration of glucose might have led to the mobilisation of fatty acids, including ALA, LA, DHA and AA from adipose tissue and the liver. It would lead to an upsurge in plasma levels of the less oxidisable AA and DHA (Leyton et al. l987) but not of readily oxidisable fatty acids, LA and ALA (Cunnane l996).

Recent study has revealed that activity of the delta-5 desaturase enzyme is enhanced in type II diabetes (Imamura et al. 2014). Up to now, there is no evidence that the activities of delta-6 and delta-5 desaturase enzymes are impaired in gestational diabetes. These enzymes are vital for the synthesis of the n-3 and n-6 LCPUFAs (Garg et al. 2017) and their impairment is often revealed by an increase in ALA and LA, and an associated decrease in their respective major metabolites DHA and AA. The data from this study do not provide support for the evidence of the impairment of either delta-6 or/and delta-5 desaturase in gestational diabetics. The GDM women had non-significantly higher AA in CPG and CE, and comparable AA (in plasma TG) and DHA in plasma CPG, CE and TG lipid fractions. Indeed, the higher AA/LA in plasma CPG and CE suggest that they might have had enhanced desaturase activity. These findings are in persistence with the previous studies that have reported enhanced AA and DHA status in plasma CPG of women with gestational diabetes (Ghebremeskel et al. 1998; Min et al. 2004; Min et al. 2006).

Contrary to the findings in plasma, the red cells of the GDM women (irrespective of their ethnic origin and dietary background) had significantly lower levels of DHA (Min et al. 2004; Min et al. 2005[a]; Min et al. 2006). We are uncertain as to why gestational diabetes had different effects on DHA levels in plasma and red cell. It is plausible that GDM reduces the incorporation of the fatty acid into red cells and other tissues. Mature red blood cells have a limited ability of synthesizing phospholipids de novo and the renewal/remodelling of red blood cell membrane phospholipids, which occurs continuously, is reliant on plasma lipid pool (Shohet 1971; Lubin 1989). Therefore, it is evident that the fatty acid moieties of red blood cell phospholipids have their origin in plasma lipids (Kleinfeld et al. 1998), in healthy individuals. In the light of these findings, our research group postulated that the incorporation of DHA from plasma into red blood cells is compromised in GDM women due to hyperglycaemia. Additionally, we postulated that the rigorous glycaemic control could improve this impairment. Based on these postulations, our research group conducted studies on GDM women supplemented with DHA-enriched formula and whose blood glucose levels were well-controlled using diet, oral hypoglycaemic agents or/and insulin. It was found that DHA level in their red blood cells phospholipids (Min et al. 2016), plasma phospholipids and neutral lipids (this study) was enhanced.

The loss of maternal plasma and red cell DHA level at the end of pregnancy has been considered as a physiological response to pregnancy. However, this phenomenon was not observed in healthy pregnant women supplemented with fish or fish oil (Miles 2011; Escolano-Margarit et al. 2013), and women from high fish and seafood-consuming communities (Luxwolda et al. 2012). Consistent with these findings, in the current study, the plasma DHA level did not decline at the end of pregnancy in the gestational diabetic women Thus, it appears that the decline of DHA level in pregnancy could be a reflection of an imbalance between maternal status and materno-foetal requirement rather than a physiological response to pregnancy.

In this study, the GDM women, who were supplemented with the placebo, had comparable DHA levels to their healthy counterparts. This may be attributable to vitamin E, which was incorporated in the placebo supplement to prevent peroxidation. Indeed, supporting evidence comes from a study by Ota (Ota et al. 2004), who reported an improvement in red cell membrane omega-6 and omega-3 fatty acid levels

after 500 mg/day of vitamin E supplementation in patients with the hepatitis C virus. However, it is worth noting that the dose that the women in our study would have consumed is minute (approximately 8 mg/d) compared with that which Ota et al. used (500mg/day).

In summary, the results of the present study show that the plasma DHA and AA levels are not compromised by gestational diabetes in pregnant women. It may be that the unaffected plasma DHA and AA levels observed in the GDM pregnant women is linked with a failure to incorporate these fatty acids into the phospholipids of the red cell membrane and impaired placental transport. Also, a considerable number of samples were collected at diagnosis, and it is plausible that the duration of the diabetes was very short to produce an obvious adverse effect on the plasma DHA and AA levels.

This study also demonstrates that DHA-enriched supplementation is effective in enhancing the maternal DHA status in pregnant women with gestational diabetes. Moreover, it shows that the decline in maternal DHA, which occurs in the final stages of gestation and was thought to be a physiological response to pregnancy, can be halted by supplementation. We suggest that the provision of a DHA supplement should be integrated with the antenatal care of pregnant women with gestational diabetes to optimize foetal development and avert maternal DHA depletion in pregnancy.

CHAPTER 4

Effect of dietary docosahexaenoic acid-enriched supplementation on plasma fatty acids profile of neonates born to the women with gestational diabetes: A randomized double-blinded placebo-controlled trial----------(100-112)

4.1 Introduction

The importance of DHA and AA in foetal life has been extensively documented (Uauy et al. 2000; Koletzko et al. 2001). They are marked as essential for optimum foetal growth and development. AA influences the foetal growth (Carlson et al. 1993; Innis 2005). DHA, a chief component of the developing CNS, is essential for visual and cognitive functions (Innis 1994; Hibbeln et al. 2007; Oken and Bellinger 2008; Makrides et al. 2010). Also, based on animal models of obesity and cell culture studies, it is postulated that DHA might be a potent anti-adiposity agent (Ruzickova et al. 2004) and enhance glucose utilization by modulating insulin secretion and action (Ramanadham et al. 2002; Gonzalez-Periz et al. 2009).

Of these two LCFUFAs, DHA is considered to be the most limiting nutrient in pregnancy and lactation, because it is scarce or absent in land animal and plant food sources. Moreover, the synthesis of DHA from the parent compound α-linolenic acid is inefficient. In a normal pregnancy, DHA is preferentially transferred by placental selection from maternal to placental circulation. However, recent studies have reported that the placental uptake and transfer of DHA is impaired in pregnancy complicated by GDM (Araujo et al. 2013; Pagan et al. 2013). Studies also show that lower values of DHA and other LCPUFAs were observed in the cord blood of neonates born to GDM mothers (Min et al. 2005[a]; Thomas et al. 2005). There is no published data whether or not the impact of this impairment could be ameliorated by DHA supplementation. This chapter presents the findings of a randomized, double-blinded, placebo-controlled trial in which the pregnant women with GDM were supplemented with DHA-enriched formula or high oleic acid sunflower seed oil. We aimed to measure the plasma phospholipids and neutral lipids DHA levels in the cord blood of neonates born to GDM women.

4.2 Subjects and methods

Subjects and methods are given in detail in **Chapter 2.**

4.3 Statistical analysis

The data are expressed as mean ± standard deviation (SD), median (range) and number of occurrence, n (%) as appropriate. Statistical significance was assumed at $P < 0.05$ unless otherwise stated. One way ANOVA was used to compare the difference in;

- Anthropometric measurements between babies born to healthy pregnant and GDM women (placebo and active-treatment groups)
- Plasma fatty acids composition of cord blood of the babies born to healthy pregnant and GDM women (placebo and active-treatment groups)

A pairwise comparison was performed using Tukey's honest significant difference (HSD, for equal variance) and Games-Howell (for unequal variance) tests for each dependent variable separately when the F-ratio was significant ($P < 0.05$). All statistical analysis were carried out with IBM SPSS Statistics version 24 (IBM Corporation, USA).

4.4 Results

4.4.1 Clinical characteristics of the neonates

The neonatal anthropometric data were obtained from babies born to 85 GDM (active-treatment group n = 44 and placebo-group n = 41) and 20 healthy pregnant (active-treatment group n = 6 and placebo-group n = 14) women. The detailed anthropometric data and pregnancy out comes of the neonates are given in **Table 4.1**. Of the two GDM women in placebo group, one delivered a stillborn baby and the other one had miscarriage. One baby was born with cleft lip and palate in GDM active-treatment group. There was no difference in the mean gestational age and the anthropometric measurements between the active-treatment and placebo groups (healthy and GDM). Although the differences were not satistically significant, but the number of babies born with low birth weight and at preterm were higher in GDM active-treatment group.

4.4.2 Plasma fatty acid composition of cord blood

This result reports the plasma choline phosphoglycerides, cholesterol esters, and triglycerides fatty acids composition of the neonatal cord blood from GDM (active-treatment group n = 44, placebo group n = 41) and healthy pregnant women (active-treatment group, n = 6; placebo group, n = 14), at delivery.

4.4.2.1 Plasma choline phosphoglycerides (CPG)

Percentages of the major fatty acids of plasma CPG are presented in **Table 4.2**. All the groups (GDM placebo-, active-treatment and healthy placebo-, active-treatment) had comparable levels of saturated and monounsaturated fatty acids. The cord blood plasma of GDM active-treatment group had low levels of AA (20:4n-6; $P < 0.01$) compared to the cord blood plasma of healthy placebo-group. Σ n-3 levels were high in healthy active-treatment group when compared to GDM placebo and GDM active-treatment groups ($P < 0.05$). Although it did not reach the level of a statistical significance, the mean percentage of DHA (22:6n-3) was also high in the healthy active-treatment group when compared to GDM placebo and GDM active-treatment groups.

4.4.2.2 Plasma cholesterol esters (CE)

Mean fatty acid composition of the cord plasma CE is presented in **Table 4.3**. All the groups (GDM placebo-, active-treatment and healthy placebo-, active-treatment) had comparable levels of saturated and monounsaturated fatty acids. Similar to the CPG fraction, the cord blood plasma of GDM active-treatment group had low levels of AA (20:4n-6; $P < 0.01$) compared to the cord blood plasma of healthy placebo-group. DHA (22:6n-3) and Σ n-3 levels were high in healthy active-treatment group as compared to GDM active-treatment group ($P < 0.05$).

4.4.2.3 Plasma triglycerides (TG)

Fatty acid composition of the cord plasma TG is given in **Table 4.4**. The levels of saturated and monounsaturated fatty acids were comparable between all the groups. Like CPG and CE fractions, although not significantly, the cord blood plasma of GDM active-treatment group had low levels of AA (20:4n-6; $P > 0.05$) compared to the cord blood plasma of healthy placebo-group. The cord blood plasma of GDM active-treatment group had low levels of adrenic acid (22:4n-6; $P < 0.05$) compared to the cord blood plasma of healthy placebo-group.

EPA (20:5n-3) was high in healthy active-treatment group when compared to GDM placebo ($P < 0.05$). The mean percentages of DHA (22:6n-3) and Σ n-3 fatty acids were also high in the healthy active-treatment group compared to GDM placebo and GDM active-treatment groups, though it did not reach the level of significance.

Within the GDM active supplemented group, the DHA levels in plasma CPG, CE and TG of neonates whose mothers had less than 10 weeks (on average) of supplementation did not differ significantly from those supplemented for a longer period.

Table 4.1. Pregnancy outcomes and anthropometric data of neonates[*].

		GDM women		Healthy pregnant women	
		Active- group	Placebo-group[a]	Active- group	Placebo-group
Live births (*n*)[b]		61	56	9	14
Gestation at delivery (weeks)[c]		38.0 (33.0-41.0)	38.0 (34.0-40.0)	39.0 (34.0-41.0)	38.0 (35.0-42.0)
Supplementation duration (weeks)[c]		10 (4.0-22.0)	10.0 (4.0-22.0)	11.0 (9.0-21.0)	12.0 (8.0-24.0)
Gender of neonates (*n*)[d]					
Male		30 (49.2)	32 (57.1)	6 (66.7)	8 (57.1)
Female		31 (50.8)	24 (42.9)	3 (33.3)	6 (42.9)
Delivery method (*n*)[d]					
Vaginal	Spontaneous	16 (26.2)	13 (23.2)	6 (66.7)	5 (35.7)
	Assisted	2 (3.3)	0 (0.0)	0 (0.0)	1 (7.1)
	Induced	11 (18.0)	15 (26.8)	0 (0.0)	1 (7.1)
Caesarean section	Elective	10 (16.4)	11 (19.6)	1 (11.1)	2 (14.3)
	Emergency	22 (36.1)	17 (30.4)	2 (22.2)	5 (35.7)
Preterm birth (*n*)[d e]		12 (19.7)	6 (10.7)	1 (11.1)	1 (7.1)
Late preterm birth (*n*)[d f]		11 (18.0)	6 (10.7)	1 (11.1)	1 (7.1)
Low birth weight (*n*)[e g]		7 (11.5)	3 (5.4)	2 (22.2)	2 (14.3)
Macrosomia (*n*)[d h]		3 (4.9)	2 (3.6)	1 (11.1)	0 (0.0)
Congenital defect (*n*)[d i]		1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)
Neonatal hypoglycemia (*n*)[d]		2 (3.3)	2 (3.6)	0 (0.0)	0 (0.0)
Anthropometric measurement[j]					
Weight (kg)		3.2 ± 0.6	3.1 ± 0.4	3.1 ± 0.7	3.0 ± 0.5
Length (cm)		49.2 ± 7.7	50.1 ± 5.6	50.0 ± 4.7	49.5 ± 3.4
Head circumference (cm)		33.8 ± 1.7	33.7 ± 2.4	33.8 ± 0.8	33.8 ± 1.3
Shoulder circumference (cm)		36.3 ± 5.4	35.9 ± 5.5	37.1 ± 1.3	36.0 ± 1.2
Mid-arm circumference (cm)		11.9 ± 2.1	11.5 ± 2.7	10.9 ± 0.7	11.0 ± 0.6
Abdominal circumference (cm)		33.1 ± 4.0	33.0 ± 4.4	32.3 ± 1.1	32.3 ± 0.9

[*] The pregnancy outcome and neonatal anthropometric measurement did not differ between the groups
[a] There was one stillbirth and one miscarriage in the placebo-group (GDM women).
[b] This number included all the subjects who completed the supplementation regardless of the availability of blood samples at delivery
[c] Data are expressed as median (min-max)
[d] Data expressed as number of subjects (%)
[e] Preterm birth; born less than 37 weeks of gestation
[f] Late-preterm birth; born between 34 0/7 weeks and 36 6/7 weeks of gestation.
[g] Low birth weight; birth weight less than 2.5kg.
[h] Macrosomia; body weight greater than 4kg.
[i] One baby from the active-group (GDM women) was born with cleft lip and palate.
[j] Data are expressed as mean ± SD. The measurement for one baby from the active-group (GDM women) was not obtained.

Table 4.2. Mean (±SD) percent fatty acid composition of cord plasma choline phosphoglycerides at delivery.

Fatty acids	**Healthy** (n = 20)		**GDM** (n = 85)	
	Placebo group (n = 14)	**Active group** (n = 6)	**Placebo group** (n = 41)	**Active group** (n = 44)
14:0	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
16:0	28.7 ± 2.1	29.0 ± 2.5	30.2 ± 2.2	30.3 ± 2.2
18:0	13.5 ± 2.2	13.9 ± 1.2	13.3 ± 1.4	13.3 ± 1.9
20:0	0.1 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.04 ± 0.01
22:0	0.1 ± 0.1	0.1 ± 0.04	0.1 ± 0.1	0.1 ± 0.1
24:0	tr	tr	tr	tr
Σ Saturates	43.2 ± 1.9	44.1 ± 2.4	44.5 ± 2.5	44.6 ± 2.3
16:1n-7	0.8 ± 0.2	0.7 ± 0.2	0.9 ± 0.3	0.9 ± 0.4
18:1n-7	2.7 ± 0.5	2.7 ± 0.4	2.7 ± 0.4	2.7 ± 0.5
18:1n-9	8.4 ± 1.5	8.9 ± 1.5	9.0 ± 1.6	9.2 ± 1.7
24:1n-9	0.05 ± 0.03	0.04 ± 0.01	0.05 ± 0.04	0.05 ± 0.04
Σ Monoenes	11.8 ± 1.8	12.4 ± 1.5	12.7 ± 1.7	12.9 ± 2.2
18:2n-6	8.7 ± 2.0	10.1 ± 2.4	10.0 ± 3.1	10.5 ± 3.0
18:3n-6	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:2n-6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.3
20:3n-6	5.5 ± 1.0	4.6 ± 1.4	5.1 ± 1.3	5.3 ± 1.2
20:4n-6	20.3 ± 2.5	17.4 ± 2.2	18.1 ± 3.2	16.8 ± 3.1^{xx}
22:4n-6	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.1
22:5n-6	0.8 ± 0.3	0.6 ± 0.4	0.7 ± 0.3	0.6 ± 0.3
Σ n-6	36.3 ± 2.6	33.7 ± 3.9	34.9 ± 3.3	34.2 ± 3.3
18:3n-3	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:5n-3	0.4 ± 0.3	0.8 ± 0.7	0.5 ± 0.4	0.5 ± 0.3
22:5n-3	0.5 ± 0.2	0.8 ± 0.4	0.5 ± 0.2	0.4 ± 0.2
22:6n-3	6.5 ± 1.9	7.4 ± 2.7	5.4 ± 1.6	5.8 ± 1.7
Σ n-3	7.6 ± 2.2	9.4 ± 3.3$^{=\infty}$	6.6 ± 1.9	6.9 ± 1.9

tr - trace
Healthy placebo versus GDM active group: $^{x}P < 0.05$, $^{xx}P < 0.01$
Healthy active versus GDM placebo group: $^{=}P < 0.05$
Healthy active versus GDM active group: $^{\infty}P < 0.05$

Table 4.3. Mean (±SD) percent fatty acid composition of cord plasma cholesterol esters at delivery.

Fatty acids	**Healthy** (n = 20)		**GDM** (n = 85)	
	Placebo group (n = 14)	**Active group** (n = 6)	**Placebo group** (n = 41)	**Active group** (n = 44)
14:0	0.2 ± 0.1	0.5 ± 0.3	0.3 ± 0.2	0.3 ± 0.3
16:0	14.6 ± 2.6	17.1 ± 2.8	16.7 ± 2.7	16.0 ± 2.6
18:0	2.2 ± 0.6	2.6 ± 0.9	2.7 ± 0.9	3.0 ± 0.9
20:0	0.04 ± 0.01	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.03
22:0	0.1 ± 0.02	0.1 ± 0.04	0.1 ± 0.04	0.1 ± 0.04
24:0	tr	tr	tr	tr
Σ Saturates	18.5 ± 3.3	23.0 ± 4.1	21.3 ± 3.5	21.1 ± 3.6
16:1n-7	4.3 ± 1.3	5.2 ± 1.8	5.7 ± 1.7	5.6 ± 2.0
18:1n-7	3.6 ± 0.6	3.6 ± 0.7	3.4 ± 0.6	3.4 ± 0.8
18:1n-9	27.1 ± 2.3	24.9 ± 3.4	28.5 ± 3.9	29.2 ± 5.5
24:1n-9	0.3 ± 0.2	0.3 ± 0.3	0.2 ± 0.1	0.3 ± 0.2
Σ Monoenes	35.2 ± 3.0	34.1 ± 2.6	37.9 ± 4.8	38.6 ± 7.3
18:2n-6	23.6 ± 8.4	21.5 ± 2.8	21.7 ± 6.6	22.3 ± 8.6
18:3n-6	0.7 ± 0.2	0.6 ± 0.2	0.8 ± 0.2	0.9 ± 0.5
20:2n-6	0.1 ± 0.01	0.04 ± 0.01	0.1 ± 0.1	0.1 ± 0.05
20:3n-6	1.7 ± 0.5	1.5 ± 0.2	1.4 ± 0.4	1.4 ± 0.4
20:4n-6	16.4 ± 4.2	14.7 ± 1.4	13.3 ± 3.4	$11.8 \pm 3.5^{\times\times}$
22:4n-6	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
22:5n-6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2
Σ n-6	42.9 ± 5.4	38.6 ± 3.3	37.6 ± 6.1	36.9 ± 9.0
18:3n-3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.2
20:5n-3	0.4 ± 0.3	0.7 ± 0.8	0.4 ± 0.3	0.3 ± 0.2
22:5n-3	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
22:6n-3	1.3 ± 0.6	$1.7 \pm 0.9^{\infty}$	1.1 ± 0.4	1.0 ± 0.6
Σ n-3	2.0 ± 0.9	$2.9 \pm 1.5^{\infty}$	1.9 ± 0.8	1.7 ± 0.9

tr - trace
Healthy placebo versus GDM active group: $^{\times}P < 0.05$, $^{\times\times}P < 0.01$
Healthy active versus GDM active group: $^{\infty}P < 0.05$

Table 4.4. Mean (±SD) percent fatty acid composition of cord plasma triglycerides at delivery.

Fatty acids	**Healthy** (n = 20)		**GDM** (n = 85)	
	Placebo group (n = 14)	**Active group** (n = 6)	**Placebo group** (n = 41)	**Active group** (n = 44)
14:0	0.8 ± 0.3	0.6 ± 0.5	0.7 ± 0.5	0.7 ± 0.4
16:0	25.1 ± 1.8	20.5 ± 5.8	24.4 ± 4.2	24.8 ± 3.4
18:0	3.8 ± 0.7	5.2 ± 1.4	5.1 ± 2.7	4.7 ± 1.3
20:0	0.1 ± 0.1	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.2
22:0	0.1 ± 0.1	0.3 ± 0.4	0.1 ± 0.1	0.1 ± 0.1
24:0	tr	tr	tr	tr
Σ Saturates	31.0 ± 1.8	28.7 ± 6.4	31.6 ± 5.5	31.5 ± 3.8
16:1n-7	4.5 ± 1.6	3.3 ± 1.4	4.6 ± 1.5	4.2 ± 1.5
18:1n-7	3.5 ± 0.7	3.9 ± 1.2	4.0 ± 0.8	3.8 ± 0.9
18:1n-9	32.8 ± 4.9	29.0 ± 4.7	32.5 ± 3.9	33.0 ± 4.6
24:1n-9	0.03 ± 0.02	0.1 ± 0.2	0.05 ± 0.1	0.1 ± 0.1
Σ Monoenes	40.9 ± 4.9	36.5 ± 3.7	41.4 ± 4.0	41.3 ± 5.1
18:2n-6	13.1 ± 1.5	17.0 ± 3.8	13.2 ± 3.2	14.0 ± 4.5
18:3n-6	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.4 ± 0.2
20:2n-6	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.5	0.3 ± 0.1
20:3n-6	1.0 ± 0.3	1.0 ± 0.4	0.8 ± 0.3	0.8 ± 0.3
20:4n-6	4.3 ± 1.5	4.9 ± 2.4	3.9 ± 1.8	3.3 ± 1.4
22:4n-6	0.9 ± 0.3	0.7 ± 0.4	0.7 ± 0.3	0.5 ± 0.2$^{\times}$
22:5n-6	1.2 ± 0.6	1.3 ± 1.1	1.2 ± 0.7	1.0 ± 0.5
Σ n-6	21.4 ± 3.1	26.1 ± 6.3	20.8 ± 4.9	20.4 ± 4.6
18:3n-3	0.6 ± 0.3	0.7 ± 0.5	0.6 ± 0.4	0.6 ± 0.3
20:5n-3	0.3 ± 0.3	0.7 ± 0.5$^{=}$	0.3 ± 0.2	0.4 ± 0.3
22:5n-3	0.6 ± 0.4	0.9 ± 0.6	0.4 ± 0.2	0.4 ± 0.3
22:6n-3	3.2 ± 1.9	4.5 ± 2.4	2.9 ± 1.4	3.3 ± 1.8
Σ n-3	5.5 ± 2.7	7.6 ± 3.4	5.1 ± 1.7	5.6 ± 2.4

tr - trace
Healthy placebo versus GDM active group: $^{\times}P < 0.05$
Healthy active versus GDM placebo group: $^{=}P < 0.05$

4.5 Discussion and conclusion

Although not statistically significant, this study indicates that the plasma n-3 and n-6 LCPUFAs in neonates may be adversely affected by gestational diabetes. A trend of reduced levels of DHA and AA were consistently observed in the cord plasma (CPG, CE and TG) of the GDM women who were supplemented with placebo compared with their healthy counterparts who received the placebo. These findings are consistent with previous report showing reduced levels of the two fatty acids in foetal plasma phospholipids and neutral lipids (Min et al. 2005[a]; Thomas et al. 2005).

Moreover, in this study, the women with GDM, who were supplemented with the placebo, had relatively equivalent DHA and AA compared with their healthy counterparts who received the placebo (discussed in Chapter 3). To support this, there is evidence to indicate that DHA and AA levels are elevated or relatively equivalent to their control groups in plasma phospholipids (Ghebremeskel et al. 1998; Wijendran et al. 1999; Min et al; 2004; Thomas et al. 2004; Min et al. 2005[a]; Min et a. 2006) and neutral lipids (Thomas et al. 2004; Min et al. 2005[a]; Min et al. 2006) of gestational diabetic women. The reduction in DHA and AA in neonatal cord plasma of the gestational diabetic women was intriguing since the nutrients are preferentially transferred from the expectant mother to her foetus (Crawford 2000[b]; Vriese et al. 2002; Gil-Sanchez et al. 2010).

The influx of n-3 and n-6 fatty acids from mother to the foetus is dependent on maternal status and placental efficiency. It is reported that dietary linoleic acid correlates with plasma or serum triglyceride linoleic acid concentrations (Moore et al. 1977). In our study, the plasma triglyceride linoleic acid concentration was significantly higher in GDM placebo women than healthy placebo women ($P < 0.01$). This might be a reflection of a high intake of linoleic acid by the GDM placebo group. On the other hand, however, the AA and DHA levels in neonates of the GMD placebo group were lower than in the neonates of healthy placebo group. Hence, it appears that, in spite of enhanced maternal LA status, the reduced levels of AA and DHA in the neonates of the gestational diabetic women could be a reflection of the impaired placental transfer. However, the evaluation of dietary data of these two groups could have been helpful to explain this point.

The uptake of LCPUFAs by the placenta is thought to be mediated by membrane bound cytosolic fatty acid binding proteins (FABPs) (Dutta-Roy 2000), and the rate of flux is primarily dependent on the abundance of the available binding sites (Haggarty 2002). It is conceivable that gestational diabetes may have an adverse effect on the binding capacity of FABPs. It is postulated that placental FABP polymorphisms may affect the processes involved in the selective transfer of LCPUFAs (Haggarty 2002). The other possibility is that the two fatty acids may be taken up by the placenta and retained instead of being transferred to the foetus. A study showing enhanced levels of AA and DHA in the placenta of gestational diabetic women (Bitsanis et al. 2004) seems to favour this proposition.

The data from the present study indicate that neonates born to the gestational diabetic women had a relative insufficiency of plasma DHA and AA, which are essential structural components of neuro, vascular and visual systems. This finding is significant since experimental and epidemiological evidence exhibits that intra uterine nutritional constraint is associated with impaired postnatal development and increased risk of chronic diseases in adulthood. It has been shown that a three-fold increase in DHA and AA occurs in human cerebrum and cerebellum during the third trimester. The lower levels of AA and DHA were found in neonates of low birth weight, small head circumference (Leaf et al. 1992[b]) and those who suffered from intrauterine growth restriction (Vilbergsson 1994). It has been shown that a higher intake of n-3 fatty acids during pregnancy is associated with gestational length and cerebral maturation in the new-born (Helland et al. 2001). A positive effect of n-3 fatty acid particularly DHA, has been recognized in insulin sensitivity (Borkman et al. 1993; Storlien et al. 1996; Clore et al. 2000), obesity (Von Kries et al. 1999) and high blood pressure (Forsyth et al. 2003).

It has been found consistently that maternal supplementation with high DHA during normal pregnancy results in enhanced DHA status in neonates (Adriana et al. 1995; Dunstan et al. 2004; Susanne et al. 2007). Recently, a report was published showing enrichment of cord blood DHA induced by maternal supplementation in pregnancy complicated by type II diabetes (Min et al. 2014). In current study, however, the neonates of the DHA-supplemented GDM women did not show the increased level of the nutrient. The exact reason behind this interesting finding is not clear since type II diabetes and GDM share many similar metabolic features and both

groups of women were supplemented daily with 600mg of DHA. However, impaired placental uptake (Araujo et al. 2013; Pagan et al. 2013), materno-foetal transfer of DHA (Pagan et al. 2013) and significant placental abnormalities have been reported more in pregnancies complicated by GDM than in type I and type II diabetes (Salge et al. 2012). Also, it is imperative to note that outcome of the current study could have been affected by the shorter supplementation duration (median 10 weeks) as compared to the study mentioned earlier (Min et al. 2014) on pregnant women with type II diabetes who were supplemented for longer duration (median 26 weeks).

Increased intake of long chain omega-3 fatty acids during pregnancy resulted in reduced number of preterm births by prolonging gestation period (Olsen et al. 1992; Olsen and Secher 2002; Klebanoff et al. 2011; Carlson et al. 2013). Likewise, the same has been observed in a small number of pregnant women with type II diabetes (Min et al. 2014). To the contrary, though statistically insignificant, the number of preterm births in the present study was higher in the women of GDM active-group. To look for the possible rationale for this finding, past obstetric histories of the women who delivered prematurely were examined. 8 out of 12 GDM women in the active-supplemented group and 4 out of 6 from GDM placebo-group who delivered prematurely had obstetric complications such as pregnancy induced hypertension, gestational diabetes and miscarriages in their previous pregnancies. Therefore, the number of preterm births encountered in the GDM women of both groups might be explained by the predisposition of these women to a recurrent preterm delivery and due to genetic-environmental factors or clinical/subclinical medical conditions rather than the effect of supplementation.

It is suggested that the supplementation of DHA during pre- and postnatal periods promotes adiposity in infants (Groh-Wargo et al. 2005; Lucia et al. 2007) and pre-school children (Donahue et al. 2011; Pedersen et al. 2012). We have evaluated whether a similar effect was present in our study using shoulder, mid-arm and abdomen circumference measurements as indirect indicators of adiposity. No difference was observed in the values of aforementioned parameters between the neonates born to the GDM active and placebo groups of mothers. This finding was not a surprise for us because the maternal supplementation did not improve foetal DHA status.

The current investigation, consistent with the previous studies mentioned-above, shows an abnormal LCPUFAs status in the healthy new-born babies of gestational diabetic women. Due to the importance of these nutrients, this abnormality may have untoward effects on foetal growth and development as well as health of an individual in adulthood. There are studies available providing evidence of speech and reading impairments, developmental concerns, and lower IQ in babies of pre-gestational and gestational diabetic women (Rizzo et al. 1991; Rizzo et al. 1995; Silverman et al. 1998; Ornoy et al. 2001). It has also been proved that intrauterine exposure to diabetes is a positive predictive factor for the development of insulin resistance and early onset type II diabetes in the offspring (Dabelea et al. 2000; Sobngwi et al. 2003).

This double-blinded, placebo-controlled, randomized study has demonstrated that DHA supplementation is effective in enhancing maternal DHA status of GDM women but not their foetal DHA status. The failure in improving the foetal DHA status by supplementation is of some concern because the babies born to GDM women are with a lower level of the nutrient. This condition is considered to be link with a risk of neuro-developmental deficits. It is plausible that supplementing the mother with more than 600mg of DHA may trickle down to the foetus and compensate the deficiency or an increased duration of maternal supplementation may improve the foetal DHA status. Nevertheless, we can allude that babies of gestational diabetic women, particularly those not sucking mother's milk, similar to the babies born prematurely would benefit from formula-milk fortified with a higher level of DHA.

CHAPTER 5

Role of dietary omega-3 polyunsaturated fatty acids (chiefly DHA) in the modulation of plasma antioxidant vitamins status in GDM women and their offspring----------(113-125)

5.1 Introduction

Oxidative stress refers to a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses that may lead to the tissue injury (Halliwell 1997). Oxidative stress due to elevated reactive oxygen species (ROS) has been clearly linked to type II diabetes mellitus (Baynes 1991; West 2000). However, inadequate and inconsistent research data are available on the involvement of oxidative stress in GDM, a disease of similar pathophysiology. Some studies have revealed the occurrence of high production of oxygen free radicals, supporting the postulation that gestational diabetes is associated with increased oxidative stress (Cederberg et al. 2001; Damasceno et al. 2002; White et al. 2002). However, there are still some loopholes in understanding about oxidative stress during gestational diabetic pregnancy and its reflection on neonates. GDM carries numerous risks for offspring including the high incidence of teratogenicity, as a result of cellular damage caused by the activity of free radicals (Surapaneni 2007).

It has been reported that oxidative system is impaired owing to both overproduction of free radicals and/or a defect in the antioxidant defense (Peuchant et al. 2004). The increased production of reactive oxygen species (ROS) has been attributed to protein glycation (Gillery et al. 1988; Baynes 1991) and glucose auto-oxidation in a hyperglycemic environment (Hunt et al. 1990). Impaired radical scavenger function has been attributed to the decreased activity of enzymatic and non-enzymatic scavengers. A study demonstrated an increase in biomarkers of oxidative stress and impaired antioxidant defense in diabetic patients (West 2000).

The biological effects of free radicals are normally controlled in vivo by a wide range of antioxidants, such as vitamin A, C and E, glutathione (GSH), and anti-oxidant enzymes, such as catalase, superoxide dismutase (SOD), GSH peroxidase, and GSH reductase. Vitamin E, the main liposoluble antioxidant in human beings, scavenges peroxyl-radicals produced during lipid peroxidation (Therond et al. 2000). Vitamin E can transfer its phenolic hydrogen to a peroxyl free radical of a peroxidized polyunsaturated fatty acid (PUFA), thereby breaking the radical chain reaction and preventing the peroxidation of PUFA in cellular and subcellular membrane phospholipids. Vitamin A also has the ability to react directly with reactive oxygen species (Livrea and Tesoriere 1998; Gutteridge and Halliwell 2000; Debier and

Larondelle 2005; Bettencourt 2010). It plays an important role in cellular function, development and maintenance of normal visual acuity (Maden 2000; Smith and Steinemann 2000). Carotenoids also have antioxidant activity. β-Carotene is reported to quench singlet molecular oxygen effectively (Krinsky 1989).

Treatment with antioxidants may prevent or reverse abnormalities associated with diabetes and its complications. Several studies have shown that dietary supplements containing vitamins and/or minerals prevent or, at least, mitigate the organic deterioration caused by increased oxidative stress in diabetics (Guerra 2001; Ylonen et al. 2003). There is a general notion that omega-3 PUFA might deteriorate antioxidant capacity. Nonetheless, no consensus has been reached on this subject as shown in **Table 5.1**. It has been argued that excessive intake of omega-3 PUFA may affect antioxidant status and enhance the susceptibility to oxidative damage (Cho and Choi 1994; Allard et al. 1997; Wander and Du 2000; Yilmaz et al. 2002; Grundt et al. 2003). Some investigators could not find any changes in the antioxidant status in humans and rats treated with omega-3 fatty acid-rich diet (Hansen et al. 1998; Ando et al. 1998; Nordoy et al. 1998). However, other researchers have demonstrated that treating diabetic patients (Kesavulu et al. 2002) or gestational diabetic rats and their offspring (Yessoufou et al. 2006) with omega-3 fatty acids significantly improves their antioxidant status. Owing to the effects of omega-3 LCPUFA stated above, we tested the hypothesis whether supplementation with dietary omega-3 is implicated in prevention and protection against free radical production or oxidative stress associated with lipid peroxidation in GDM.

To our knowledge, no research study has directly investigated the role of DHA-enriched supplementation on plasma antioxidant vitamins status in gestational diabetic women and their neonates. Therefore, it was thought worthwhile to undertake the current study to evaluate the antioxidant status of gestational diabetic women (who had enhanced LCPUFAs status due to DHA-enriched supplementation) and their new-borns. Maternal and cord plasma α-tocopherol, retinol and β-carotene levels were estimated to evaluate the natural antioxidant status.

Table 5.1. Effects of omega-3 fatty acids on antioxidant status as reported by various investigators (adapted from Yessoufou et al. 2006).

Omega-3 PUFA level in the diet	Species	Antioxidant status	References
10% of diet (considered as excessive)	Diabetic rats	Decreased	Cho and Choi 1994
EPA: 2.5 g/day; DHA: 1.8 g/day	Healthy humans	Decreased	Wander and Du 2000
6.26 g/day for 6 weeks	Healthy humans	Decreased	Allard et al. 1997
850–882 mg/day (EPA + DHA) for 1 year	Patients with myocardial infarction	Decreased	Grundt et al. 2003
Fish oil	Diabetic rats	Decreased	Yilmaz et al. 2002
4 g/day (n-3) PUFA for 5 weeks	Healthy humans	Unchanged	Hansen et al. 1998
n-3 fatty acid-rich diet (fish oil)	Rats	Unchanged	Ando et al. 1998
4 g/day (DHA or EPA)	Hyperlipidemic patients	Unchanged	Nordøy et al. 1998
EPA: 1.08 g/day; DHA: 0.72 g/day for 2 months	Diabetic humans	Improvement	Kesavulu et al. 2002
2.1% of diet	Diabetic rats	Improvement	Yessoufou et al. 2006[a]
400mL/day of energy drink (DHA 320mg, EPA 72mg, vit. E 6mg, vit. C 36mg vit. D 3µg) for 7 months	Normal pregnant women and their infants	Improvement	Kajarabille et al. 2017

5.2 Subjects and methods

Subjects and methods are described in detail in **Chapter 2.**

5.3 Statistical analysis

The data are expressed as mean ± standard deviation (SD), median and inter-quartile range (IQR) as appropriate.

At baseline;

- Independent samples t-test was run to explore the significant differences in plasma alpha-tocopherol levels between; (**a**) healthy pregnant and GDM women (**b**) GDM placebo- and GDM active group.
- Mann Whitney U test was run to explore the significant differences in plasma retinol and beta-carotene levels between; (**a**) healthy pregnant and GDM women (**b**) GDM placebo- and GDM active group.

At delivery;

- Independent samples t-test was used to explore the significant differences in the maternal and cord plasma alpha-tocopherol levels between GDM-placebo and GDM-active treatment group.
- Mann Whitney U test was used to explore the significant differences in the maternal and cord plasma retinol and beta-carotene levels between GDM-placebo and GDM-active treatment group.

Within-group baseline and delivery comparison, the maternal plasma alpha-tocopherol, retinol and beta-carotene levels were compared using Wilcoxon Signed Ranks test. Statistical significance was assumed at $P < 0.05$ unless otherwise stated. All statistical analysis were carried out with IBM SPSS Statistics version 24 (IBM Corporation, USA).

5.4 Results

The antioxidant data was obtained from 85 GDM (active-treatment group n = 44 and placebo-group n = 41) and 13 healthy pregnant (baseline data) women. The antioxidant status of the subjects was evaluated by estimation of the plasma α-tocopherol (vitamins E), retinol (vitamin A) and β-carotene levels.

5.4.1 Maternal and neonatal plasma antioxidant vitamins levels

5.4.1.1 Plasma α-tocopherol levels

At baseline, plasma α-tocopherol levels were comparable between healthy pregnant and GDM women (**Figure 5.1**). Maternal plasma α-tocopherol levels were comparable between GDM placebo and GDM active-treatment group, at baseline and delivery ($P > 0.05$). At delivery, no significant difference was found in cord plasma α-tocopherol levels between GDM placebo and active-treatment groups. Plasma α-tocopherol levels at baseline and delivery are presented in **Table 5.2**.

Within-group baseline and delivery comparison in plasma α-tocopherol revealed no significant changes in both GDM active-treatment and placebo groups ($P > 0.05$). **Table 5.5** shows plasma α-tocopherol levels compared within-group baseline and delivery.

Table 5.2. Maternal and cord plasma α-tocopherol levels (Mean ± SD) at baseline and delivery.

α-tocopherol (mg/dl)	**Baseline**	**Delivery-maternal**	**Delivery-cord**
GDM placebo group (n = 41)	1.3 ± 0.3	1.3 ± 0.4	0.3 ± 0.1
GDM active group (n = 44)	1.3 ± 0.4	1.4 ± 0.4	0.3 ± 0.1

5.4.1.2 Plasma retinol levels

Plasma retinol levels at baseline and delivery are presented in **Table 5.3.** At baseline, plasma retinol levels were comparable between healthy pregnant and GDM women (**Figure 5.1**), and between GDM placebo- and GDM active-treatment groups ($P > 0.05$). At delivery, no significant difference was found in plasma retinol levels of maternal (GDM placebo vs. active-treatment group) and cord blood (GDM placebo vs. active-treatment group).

Within-group baseline and delivery comparison, the plasma retinol levels were reduced significantly in GDM placebo ($P < 0.001$) and GDM active-treatment groups ($P < 0.001$). **Table 5.5** presents within-group baseline and delivery comparison in plasma retinol levels.

Table 5.3. Maternal and cord plasma retinol levels (Median (IQR)) at baseline and delivery.

Retinol (μg/dl)	**Baseline**	**Delivery-maternal**	**Delivery-cord**
GDM placebo group (n = 41)	25.0 (8.4)	21.1 (6.6)	13.3 (6.3)
GDM active group (n = 44)	23.4 (7.5)	18.0 (6.5)	12.0 (5.7)
Mann-Whitney U	426	451	454
P - value	0.318	0.409	0.432

5.4.1.3 Plasma β-carotene levels

At baseline, plasma β-carotene levels were comparable between healthy pregnant and GDM women (**Figure 5.1**), and between GDM placebo- and GDM active-treatment groups ($P > 0.05$). At delivery, no significant difference was found in plasma β-carotene levels of maternal (GDM placebo vs. active-treatment group) and

cord blood (GDM placebo vs. active-treatment group). **Table 5.4.** shows plasma β-carotene levels at baseline and delivery.

Within-group baseline and delivery comparison, a significant decrease in plasma β-carotene levels was seen in GDM active-treatment group ($P < 0.01$). GDM placebo group exhibited no significant changes in plasma β-carotene levels. Within-group baseline and delivery comparison of plasma β-carotene levels are given in **Table 5.5.**

Table 5.4. Maternal and cord plasma β-carotene levels (Median (IQR)) at baseline and delivery.

β-carotene (μg/dl)	**Baseline**	**Delivery-maternal**	**Delivery-cord**
GDM placebo group (n = 41)	18.9 (28.0)	16.1 (24.6)	1.1 (2.1)
GDM active group (n = 44)	14.5 (28.1)	11.1 (14.1)	1.0 (0.9)
Mann-Whitney U	466	425	508
P - value	0.649	0.241	0.946

Table 5.5. Changes in plasma antioxidant vitamins level (Median (IQR)) between the baseline and delivery within GDM (placebo and active) groups.

Maternal baseline vs. delivery		**α-tocopherol** (mg/dl)	**Retinol** (μg/dl)	**β-carotene** (μg/dl)
GDM placebo group (n = 41)	Baseline	1.3 (0.4)	25.0 (8.4)	18.9 (28.0)
	Delivery-maternal	1.3 (0.6)	21.1 (6.6)***	16.1 (24.6)
GDM active group (n = 44)	Baseline	1.3 (0.4)	23.4 (7.5)	14.5 (28.1)
	Delivery-maternal	1.4 (0.5)	18.0 (6.5)***	11.1 (14.1)**

Maternal baseline versus delivery within the group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

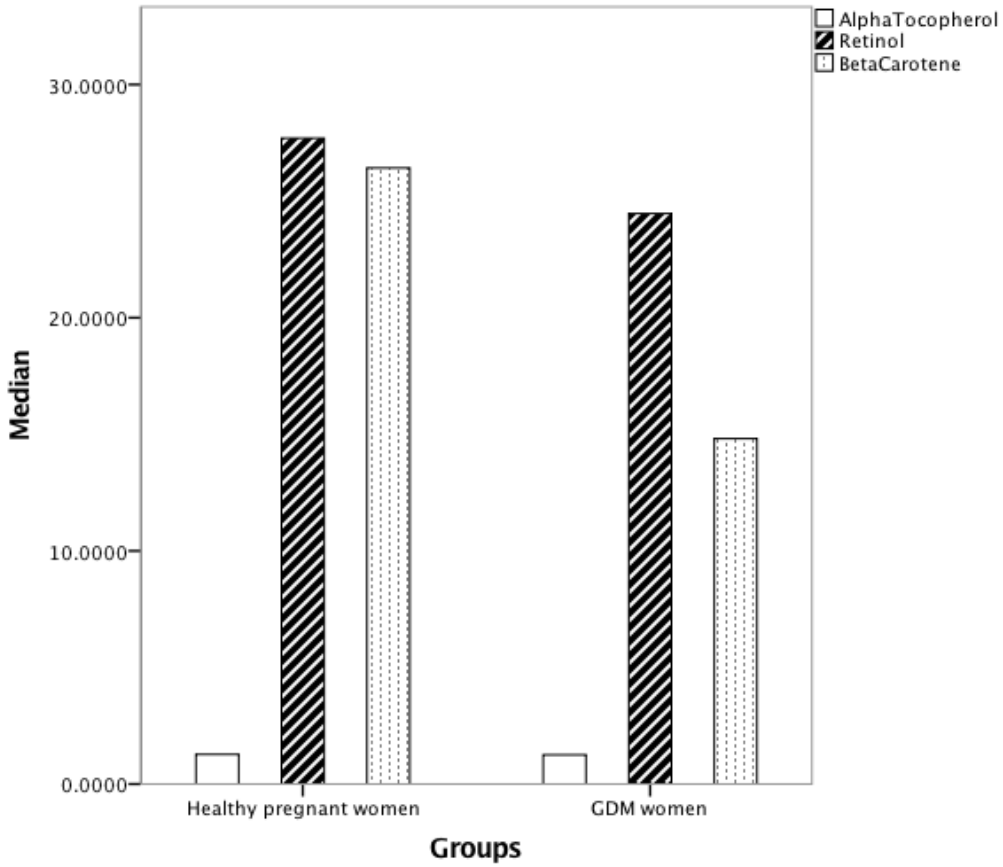

Figure 5.1. A bar graph showing differences in plasma α-tocopherol (1.3 (0.4) vs. 1.3 (0.3)), retinol (27.7 (8.8) vs. 24.2 (8.5)) and β-carotene (26.4 (50.5) vs. 14.8 (27.9)) levels between healthy pregnant (n = 13) and GDM women (n = 85), at baseline. The levels of the vitamins were comparable between the groups ($P > 0.05$). values are given as healthy pregnant vs. GDM women (median (IQR)).

5.5 Discussion and conclusion

There are few studies dealing with the status of vitamin A, E and β-carotene in maternal and cord blood of gestational diabetic women. The majority of these studies have controversial findings. Therefore, we evaluated the aforementioned vitamins status in maternal and cord plasma samples of gestational diabetic women. In addition, the role of omega-3 LCPUFA (essentially DHA and EPA) on natural antioxidant status in gestational diabetic mothers and their offspring was also investigated.

In the current study, plasma α-tocopherol (vitamin E) level was comparable between healthy pregnant and GDM women. Similar findings have been shown in other studies (Sobki et al. 2004; Dey et al. 2008). There have been conflicting reports on plasma vitamin E concentrations in GDM. Contrary to our findings, a few studies reported that plasma vitamin E level was significantly lower in gestational diabetic women (Peuchant et al. 2004; Grissa et al. 2007). Another study showed a slight reduction in the plasma vitamin E level in GDM women (Kharb 2000). Suhail et al. (Suhail et al. 2010) also found decreased serum vitamin E concentrations in GDM women compared to the control group. On the other hand, Bates et al. (Bates et al. 1997), Resende et al. (Resende et al. 2014) and Santra et al. (Santra et al. 2003) had reported an increase in serum vitamin E in GDM women.

This unchanged vitamin E status in GDM women as found in our and some other studies seem to represent an adaptive response to high oxidative stress. This increase in oxidative stress in the diabetic state has also been reported earlier (Silvana et al. 2004). As alpha-tocopherol acts as a potent antioxidant, it is oxidized and transformed into a free radical (tocopherol), thus requiring a regeneration system which promotes the recovery of its antioxidant function. Hence, other antioxidants such as; vitamin C, reduced-glutathione and Coenzyme Q10 remove the free radical from the tocopherol molecule. Giannubilo et al. determined a significantly higher plasma level of coenzyme Q10 in late pregnancy in GDM women, compared to the control group. To elucidate this difference, the authors proposed that there is a compensatory mechanism/adoptive response in response to high oxidative stress linked with hyperglycaemia and insulin resistance in GDM women (Giannubilo et al. 2011).

The unchanged vitamin E level may also be related to metabolic changes, which occur in the maternal body due to gestational diabetes. At the end of the pregnancy, there should be decreased insulin secretion, leading to a hormonal response that make the tissues dependent on insulin to metabolize lipids in place of carbohydrates, starting a process of lipolysis (Resende et al. 2014), which culminates in the increased release of free fatty acids in the circulation (Fulop et al. 2006). Since the primary means of storage of vitamin E in the body is the adipose tissue (Herrera and Barbas 2001), this vitamin can be influenced by increased lipolysis, leading diabetic women to present with enhanced vitamin E status.

We also found that plasma retinol (vitamin A) and β-carotene levels tended to be lower in GDM women (although statistically insignificant) compared to healthy pregnant women. Again, we came across some controversial reports in regard to the status of these micronutrients. Also, the data available about vitamin A and β-carotene levels in diabetes mellitus is very limited. However, our findings are in harmony with the earlier reports showing that retinol was significantly less in GDM women as compared to their control (Peuchant et al. 2004; Suhail et al. 2010; Hekmat et al. 2014). Some others reported no change in vitamin A level in GDM women (Bates et al. 1997; Grissa et al. 2007). To the best of our knowledge, no study has yet been conducted to determine any differences in the plasma concentrations of β-carotene in GDM women. According to a study, a low β-carotene level was observed in type I diabetic women compared to the healthy controls (Azar et al. 2011). Furthermore, there are studies showing inverse association between obesity and vitamin A and β-carotene concentrations (Andersen et al. 2006; Kimmons et al. 2006). The low levels with increase obesity may result from inadequate intake and/or alterations in the nutrient metabolism since the plasma concentrations of β-carotene correlates with the consumption of vegetables and fruits. In addition, low level of vitamin A and β-carotene was found in obese pregnant women as compared to healthy pregnant women (Tomedi et al. 2013). Since obesity is a one of the known risk factors for GDM so one can speculate that the relative low vitamin A and β-carotene levels in GDM women as found in our study could be negatively associated with their high BMI.

In our study, GDM women who were supplemented with omega-3 enriched formula did not exhibit any significant difference in plasma vitamins E, A and β-carotene levels when compared with their GDM counterparts who received the

placebo, at delivery. Similar results were found when cord plasma levels of these micronutrients were compared between the groups. There is no consensus among the researchers on the antioxidant effects of omega-3 PUFA supplementation. It has been reported that excessive intake of omega-3 PUFA may affect the antioxidant status by enhancing the susceptibility to oxidative damage (Allard et al. 1997; Wander and Du 2000; Grundt et al. 2003). Whereas, some investigators could not establish any changes in the antioxidant status in humans supplemented with omega-3 PUFA rich diet (Hansen et al. 1998; Nordoy et al. 1998). Another study found that treating diabetic patients with omega-3 PUFA significantly improves their antioxidant status (Kesavulu et al. 2002). A recent study conducted in Spain showed improved antioxidant status among normal pregnant women and their infants after omega-3 LCPUFA enriched supplementation (Kajarabille et al. 2017). We are unaware of any study which evaluated the effect of omega-3 supplementation on the antioxidant status in GDM women. While looking into the conflicting results of the above studies, it was found that dose of omega-3 rich-formula and duration of supplementation were different in each study (**Table 5.1**). Since the antioxidant effects are linked with dose and the antioxidant defence status of the subjects (Shoji et al. 2009; Di Nunzio et al. 2011; Hajjaji and Bougnoux 2013), particularly in conditions of increase oxidative stress and/or impaired antioxidant defence such as GDM, one can speculate that anti-oxidant effects of omega-3 supplementation are likely to be dose and duration dependent.

Kesavulu et al. (2002) showed improved antioxidant status in non-insulin dependent type II diabetic patients. Although this study was restricted to type II diabetes, their findings would be expected to be applicable to gestational diabetes as both conditions share many common metabolic features. Although, there was a minimal difference in the duration of supplementation but the dose of omega-3 LCPUFA used in the study was much higher **(Table 5.1)** as compared to our study. Another difference was that Kesavulu et al. evaluated the enzymatic antioxidant status (GPx, CAT, SOD) whereas, we determined the non-enzymatic antioxidant status (Vitamin A, E and β-carotene).

Kajarabille et al. (2017) conducted a more comprehensive study on the antioxidant effects of omega-3 LCPUFA among normal pregnant women and their infants. Nevertheless, the studies on the antioxidant effects of omega-3 LCPUFA

supplementation in the mother and their neonates are really scarce. Although this study was restricted to normal pregnant women, however, a noteworthy aspect to consider during pregnancy (Diaz-Castro et al. 2015) is the oxidative stress which is even more worse in GDM (Suhail et al. 2010) affecting both mother and their neonates. The omega-3 supplement formula used in the study was different from our study and included Vitamin E (much higher than in our formula), C and D (**Table 5.1**). In addition, 2/3 portions of fish per week were recommended to the pregnant and lactating women based on their dietary data evaluation. Moreover, the total duration of supplementation was around 7 months which was much shorter (median 10 weeks) in our study. The study evaluated both the enzymatic and non-enzymatic components of antioxidant defense system at recruitment, delivery, 2.5 months postpartum and at 4 months postpartum between mothers and babies of both groups (supplemented group vs. control group). The findings of the study included that the levels of plasma Vitamin A, E and β-carotene were comparable between the maternal and cord plasma of both groups at delivery (in corroboration of our findings), however increased significantly at 2.5 months postpartum. These findinds further strengthen our speculation that the antioxidant effects of omega-3 PUFA are dependent on dose and supplementation duration.

In our study, the antioxidant status did not improve yet not deteriorate in GDM active-treatment group despite of the less dose and duration of omega-3 LCPUFA supplementation. Another aspect to be taken into account is that antioxidant defence system includes non-enzymatic antioxidants (vitamins A and E, β-carotene) and antioxidant enzymes such as; superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GSSG-Red) (Yessoufou and Moutairou 2011). However, this study only determined the non-enzymatic antioxidant status (vitamins A, E and β-carotene). In summary, we can allude that the moderate amounts of omega-3 LCPUFA in dietary intake for longer duration could enhance the anti-oxidant status in GDM women and their neonates. Our study has provided a base for future studies which could evaluate the effects of omega-3 LCPUFAs on the antioxidant stautus in GDM women more comprehensively taking into account all the above-mentioned factors.

CHAPTER 6

6.1 Introduction

Overweight and obesity are recognised as major public health issues among all ages and populations in Western countries (Haslam and James 2005; Song et al. 2014; Liu et al. 2015). An up to 30 % prevalence of obesity is reported among pregnant women (Callaway et al. 2006; Huda et al. 2009). A number of adverse pregnancy outcomes such as gestational diabetes mellitus, gestational hypertension, foetal death, and lager for gestational age infants are linked to obesity during pregnancy (Driul et al. 2008; Kongubol and Phupong 2011; Gaillard 2015; Starling et al. 2015). However, the mechanisms underlying these increased risks for pregnancy complications are incompletely understood.

Increase body mass index (BMI) is associated with alterations in lipid metabolism and insulin resistance during pregnancy, which may cause increased circulating free fatty acids concentrations (Nelson et al. 2010). High levels of saturated fatty acids, low levels of ω-3 PUFA and high ω-6/ ω-3 ratio have been found associated with high BMI, in adolescents and adults (Karlsson et al. 2006; Micallef et al. 2009). A previous study conducted in USA on 129 pregnant women categorised by their pre-pregnancy BMI showed that pre-pregnancy obesity is associated with lower concentrations of DHA and AA but not of EPA. This study, however, did not provide information about concentrations of other fatty acids (Tomedi et al. 2013). There is also limited data available comparing fatty acid profiles between normal weight, overweight and obese women during pregnancy. Also, we are unaware of any previous studies that have compared the maternal plasma fatty acid profile in GDM women of different weight categories. Therefore, we conducted this analysis to evaluate the difference in plasma fatty acid levels of GDM women categorised on the basis of their pre-pregnancy BMI.

6.2 Subjects and Methods

Participants were classified as normal weight, overweight and obese if their BMI were $\leq 25.0 kg/m^2$, 25.1-30.0 kg/m^2 and >30.0 kg/m^2, respectively. The details of subjects and methods are described in **Chapter 2**.

6.3 Statistical analysis

The data are expressed as mean ± standard deviation (SD), n (%) and median (range) as appropriate. Statistical significance was assumed at $P < 0.05$ unless otherwise stated. One-way analysis of variance (ANOVA) was used to compare the differences in demographic data and plasma fatty acid levels of GDM women belonging to different BMI categories. A pairwise comparison was performed using Tukey's honest significant difference (HSD, for equal variance) and Games-Howell (for unequal variance) tests for each dependent variable separately when the F-ratio was significant ($P < 0.05$). All statistical analyses were carried out with IBM SPSS Statistics version 24 (IBM Corporation, USA).

6.4 Results

6.4.1 Demographic characteristics of the participants

Table 6.1 shows the demographic characteristics of normal weight compared to overweight and obese GDM women. The obese GDM women tended to be older and of high parity, as compared to normal weight women. The education levels did not differ between the groups.

The majority of the subjects were of asian (around 60.6%) and African/Afro-Caribbean (24.6%) origin. However, the African/Afro-Caribbean origin women dominated in obese group (43.1%).

Table 6.1. Demographic characteristics of the participants.

Maternal characteristics	Normal weight (≤ 25.0 kg/m^2)	Over weight (25.1-30.0)	Obese (> 30.0)
Number of participants (*n*)	44	47	51
Gestation at recruitment (weeks)[a]	26.5 (17.0-32.0)	28.0 (17.0-34.0)	28 (17-32)
Age (years)[a]	30.0 (20-43)	32.0 (19-44)	32 (23-43)
Height (m)[b]	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Pre-pregnancy weight (kg)[b]	57.4 ± 7.1***	71.4 ± 7.9$^{===}$	89.3 ± 10.3^{+++}
Pre-pregnancy BMI (kg/m^2)[b]	23.2 ± 2.2***	28.1 ± 1.5$^{===}$	35.3 ± 3.4^{+++}
Education (*n*)[c]			
Higher education	14 (31.8)	19 (40.2)	18 (35.3)
GCSE	15 (34.1)	8 (17.0)	13 (25.5)
A-level	5 (11.4)	9 (19.1)	3 (5.9)
None	5 (11.4)	5 (10.6)	9 (17.6)
Unknown	5 (11.4)	6 (12.8)	8 (15.7)
Racial Origin (*n*)[c, d]			
Asian	36 (81.8)	31 (66.0)	19 (37.3)
African/Afro-Caribbean	3 (6.8)	10 (21.3)	22 (43.1)
Caucasian	2 (4.5)	3 (6.4)	7 (13.7)
Others	3 (6.8)	3 (6.4)	3 (5.9)
Parity (*n*)[c]			
0	22 (50.0)	15 (31.9)	18 (35.3)
1-2	18 (40.9)	23 (48.9)	17 (33.3)
≥ 3	4 (9.1)	9 (19.1)	16 (31.4)
Folic acid use (*n*)[c]	26 (59.1)	26 (55.3)	29 (56.9)
Smoker (*n*)[c]	0 (0.0)	1 (2.1)	1 (2.0)
Glucose tolerance test			
Gestation (weeks)[a]	18.0 (15-29)	22.0 (12-31)	20.0 (13-31)
Glucose at 0 min (mmol)[b]	5.4±1.3	5.4±1.3	5.9±1.8
Glucose at 120 min (mmol)[b]	9.1±1.8	9.5±1.9	9.3±1.9

[a] Data are expressed as median (mininum - maximum)
[b] Data are expressed as mean ± SD
[c] Data are expressed as number of subjects (%)
[d] We classified individuals according to the UK Home Office's classification for an individual's ethnicity which is based on person's self-defination (**African/Afro-Caribbean**: Afro-Caribbean, Black African, Black British, Caribbean; **Asian**: Bangladesh, Bengali, Indian, Pakistani, Sri Lankan; **Caucasian**: English, European, Irish, Polish; **Others**: Arab, Filipino, Latin American, North African, mixed race).
Normal weight versus overweight group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$
Normal weight versus obese group: $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$
Overweight versus obese group: $^{=}P < 0.05$, $^{==}P < 0.01$, $^{===}P < 0.001$

6.4.2 Plasma fatty acid composition in GDM women

This result reports plasma choline phosphoglycerides, cholesterol esters, and triglycerides fatty acids of the GDM women catagorised on the basis of their pre-pregnancy BMI ≤ 25.0 (n = 44), 25.1-30.0 (n = 47) and > 30.0 (n = 51). Also, Plasma DHA and AA levels of the three groups are presented in graphical form **(Figure 6.1, 6.2, 6.3)**.

6.4.2.1 In plasma CPG: Fatty acid composition of plasma CPG is given in **Table 6.2.** 20:4n-6 (AA) level was higher in over-weight ($P < 0.05$) and obese groups ($P < 0.01$) compared to normal-weight group. There was an increase in Σ n-6 fatty acids in over-weight group when compared to normal-weight group ($P < 0.05$). 18:3n-3 (α-linolenic acid) was decreased in both obese and over-weight groups than normal-weight group ($P < 0.05$). Total saturated and monounsaturated fatty acid levels were not different between the groups (all P values > 0.05).

6.4.2.2 In plasma CE: Similar to the CPG composition, 20:4n-6 (AA) level was higher in over-weight ($P < 0.05$) and obese groups ($P < 0.01$) compared to normal-weight group. 18:3n-3 (α-linolenic acid) was decreased in both obese and over-weight groups than normal-weight group ($P < 0.05$). Total saturated and monounsaturated fatty acid levels were not different between the groups (all P values > 0.05). Fatty acid composition of plasma CE is given in **Table 6.3.**

6.4.2.3 In plasma TG: 14:0 (myristic acid; $P < 0.05$) level was lower in over-weight and obese groups compared to normal-weight group. There was also a decrease in 16:1n-7 (palmitoleic acid) in over-weight group when compared to normal-weight group ($P < 0.05$). The level of 18:3n-3 (α-linolenic acid) was decreased in obese group than normal-weight group ($P < 0.05$). Fatty acid composition of plasma TG is given in **Table 6.4.**

Table 6.2. Mean (±SD) percent fatty acid composition of plasma choline phosphoglycerides in GDM women.

Fatty acids	Normal weight (n = 44)	Over weight (n = 47)	Obese (n = 51)
14:0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
16:0	30.3 ± 3.8	29.9 ± 2.9	30.3 ± 3.3
18:0	9.8 ± 1.2	10.2 ± 1.4	10.4 ± 1.2
20:0	0.04 ± 0.04	0.03 ± 0.02	0.03 ± 0.01
22:0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
24:0	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.02
Σ Saturates	41.2 ± 3.8	41.2 ± 2.1	41.7 ± 2.5
16:1n-7	0.5 ± 0.3	0.4 ± 0.2	0.4 ± 0.2
18:1n-7	1.4 ± 0.5	1.3 ± 0.3	1.4 ± 0.2
18:1n-9	9.8 ± 4.1	9.1 ± 1.8	9.4 ± 1.8
24:1n-9	0.05 ± 0.02	0.04 ± 0.02	0.05 ± 0.03
Σ Monoenes	12.0 ± 4.8	11.0 ± 2.0	11.5 ± 2.1
18:2n-6	24.9 ± 3.7	25.4 ± 2.9	24.2 ± 3.0
18:3n-6	0.1 ± 0.1	0.1 ± 0.04	0.1 ± 0.03
20:2n-6	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20:3n-6	3.5 ± 0.8	3.7 ± 1.0	3.6 ± 0.9
20:4n-6	9.8 ± 2.1	$10.9 \pm 1.9^{*}$	$11.1 \pm 1.9^{++}$
22:4n-6	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
22:5n-6	0.5 ± 0.5	0.4 ± 0.2	0.4 ± 0.2
Σ n-6	39.5 ± 3.6	$41.3 \pm 3.2^{*}$	40.1 ± 3.2
18:3n-3	0.4 ± 0.2	$0.3 \pm 0.1^{*}$	$0.3 \pm 0.1^{+}$
20:5n-3	0.8 ± 0.7	0.6 ± 0.5	0.7 ± 0.7
22:5n-3	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
22:6n-3	4.6 ± 1.1	4.1 ± 1.0	4.4 ± 1.1
Σ n-3	6.5 ± 1.7	5.7 ± 1.5	6.0 ± 1.8

Normal weight versus overweight group: $^{*}P < 0.05$
Normal weight versus obese group: $^{+}P < 0.05$, $^{++}P < 0.01$

Table 6.3. Mean (±SD) percent fatty acid composition of plasma cholesterol esters in GDM women.

Fatty acids	Normal weight (n = 44)	Over weight (n = 47)	Obese (n = 51)
14:0	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
16:0	9.6 ± 1.8	9.2 ± 1.9	9.8 ± 1.6
18:0	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
20:0	0.05 ± 0.03	0.04 ± 0.02	0.04 ± 0.02
22:0	0.03 ± 0.02	0.02 ± 0.02	0.03 ± 0.02
24:0	tr	tr	tr
Σ Saturates	12.7 ± 3.3	11.3 ± 2.9	12.3 ± 3.1
16:1n-7	1.9 ± 1.3	1.4 ± 0.8	1.5 ± 0.8
18:1n-7	1.1 ± 0.3	1.0 ± 0.2	1.1 ± 0.2
18:1n-9	15.8 ± 3.2	15.3 ± 2.3	16.2 ± 2.8
24:1n-9	0.6 ± 0.4	0.8 ± 0.3	0.7 ± 0.4
Σ Monoenes	19.4 ± 4.1	18.5 ± 2.8	19.5 ± 3.3
18:2n-6	55.4 ± 5.8	56.8 ± 5.3	54.7 ± 4.9
18:3n-6	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
20:2n-6	0.1 ± 0.02	0.1 ± 0.02	0.05 ± 0.02
20:3n-6	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.3
20:4n-6	7.9 ± 1.9	$8.9 \pm 2.0^{*}$	$9.2 \pm 2.0^{++}$
22:4n-6	0.1 ± 0.1	0.05 ± 0.03	0.04 ± 0.02
22:5n-6	0.1 ± 0.1	0.1 ± 0.04	0.1 ± 0.1
Σ n-6	64.9 ± 5.8	67.3 ± 5.3	65.4 ± 4.9
18:3n-3	0.8 ± 0.3	$0.6 \pm 0.3^{*}$	$0.6 \pm 0.3^{+}$
20:5n-3	0.8 ± 0.6	0.6 ± 0.6	0.7 ± 0.7
22:5n-3	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.03
22:6n-3	0.9 ± 0.3	0.9 ± 0.2	0.9 ± 0.3
Σ n-3	2.6 ± 0.9	2.3 ± 0.8	2.4 ± 1.0

Normal weight versus overweight group: $^{*}P < 0.05$
Normal weight versus obese group: $^{+}P < 0.05$, $^{++}P < 0.01$

Table 6.4. Mean (±SD) percent fatty acid composition of plasma triglycerides in GDM women.

Fatty acids	Normal weight (n = 44)	Over weight (n = 47)	Obese (n = 51)
14:0	1.4 ± 0.7	1.1 ± 0.7*	1.1 ± 0.6+
16:0	27.0 ± 4.3	26.0 ± 3.7	26.8 ± 3.9
18:0	2.8 ± 0.5	3.0 ± 0.7	3.1 ± 0.5
20:0	0.1 ± 0.1	0.1 ± 0.04	0.1 ± 0.02
22:0	0.04 ± 0.02	0.04 ± 0.03	0.05 ± 0.03
24:0	tr	tr	tr
Σ Saturates	32.7 ± 4.9	31.3 ± 4.4	32.3 ± 4.7
16:1n-7	2.2 ± 0.9	1.8 ± 0.7*	1.9 ± 0.9
18:1n-7	2.1 ± 0.5	2.1 ± 0.4	2.2 ± 0.4
18:1n-9	32.4 ± 5.0	34.0 ± 4.9	34.0 ± 4.9
24:1n-9	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02
Σ Monoenes	37.2 ± 5.3	38.2 ± 5.2	38.5 ± 5.2
18:2n-6	23.3 ± 6.1	23.9 ± 5.9	22.8 ± 5.3
18:3n-6	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1
20:2n-6	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
20:3n-6	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20:4n-6	1.3 ± 0.4	1.4 ± 0.4	1.4 ± 0.4
22:4n-6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
22:5n-6	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
Σ n-6	26.1 ± 6.4	26.9 ± 6.3	25.7 ± 5.7
18:3n-3	1.5 ± 0.6	1.2 ± 0.6	1.2 ± 0.5+
20:5n-3	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.3
22:5n-3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
22:6n-3	1.0 ± 0.5	0.8 ± 0.5	0.9 ± 0.7
Σ n-3	3.2 ± 1.0	2.6 ± 1.1	2.7 ± 1.3

Normal weight versus overweight group: $^{*}P < 0.05$
Normal weight versus obese group: $^{+}P < 0.05$

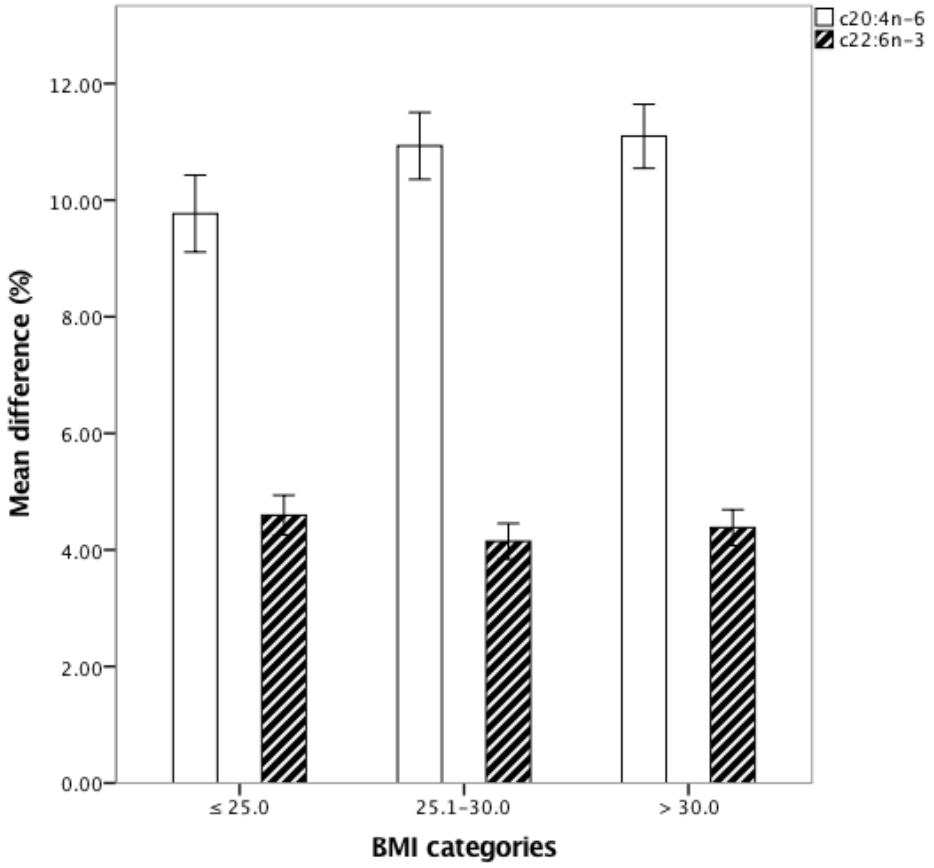

Figure 6.1. A bar graph showing AA and DHA levels in plasma CPG of GDM women belonging to different pre-pregnancy BMI categories. AA (20:4n-6) levels were higher in over-weight ($P < 0.05$) and obese ($P < 0.01$) GDM women compared to normal-weight GDM women. DHA (22:6n-3) levels were comparable between the three categories.

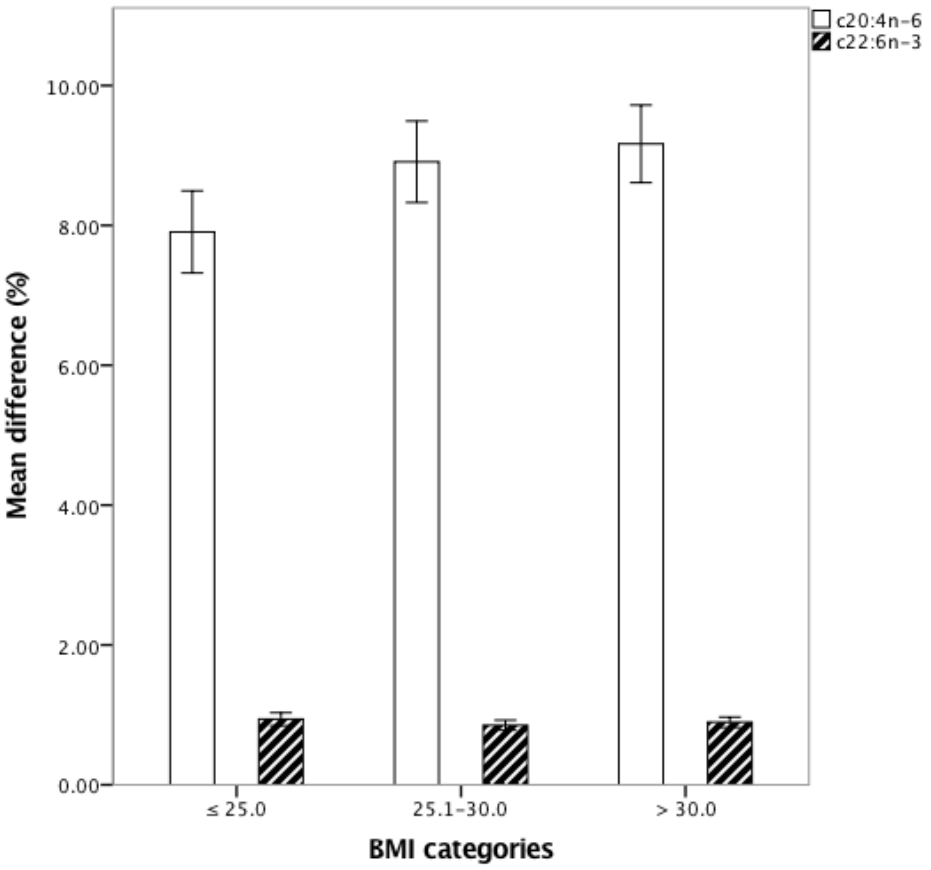

Figure 6.2. A bar graph showing AA and DHA levels in plasma CE of GDM women belonging to different pre-pregnancy BMI categories. AA (20:4n-6) levels were higher in over-weight ($P < 0.05$) and obese ($P < 0.01$) GDM women compared to their normal-weight counterparts. DHA (22:6n-3) levels were comparable between the three categories.

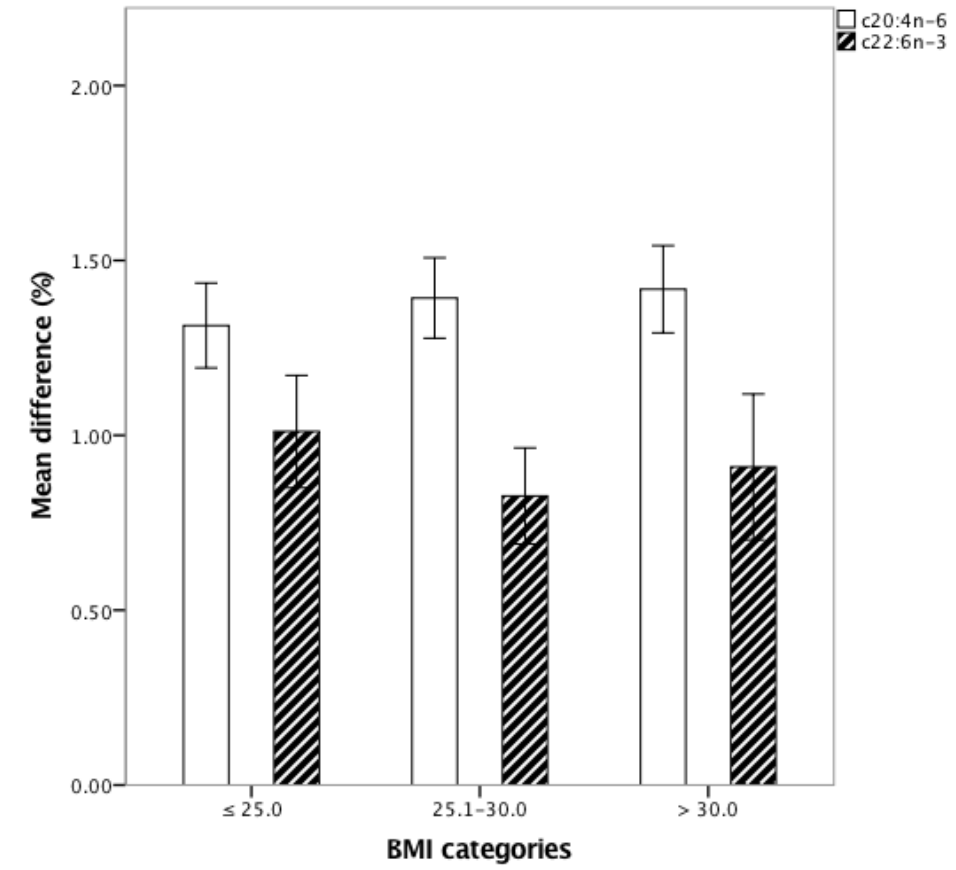

Figure 6.3. A bar graph presenting AA and DHA levels in plasma TG of GDM women categorised on the basis of their pre-pregnancy BMI. Although not significantly ($P > 0.05$), AA (20:4n-6) levels were high in both over-weight and obese GDM women compared to the GDM women of normal-weight. DHA (22:6n-3) levels were comparable between the three categories.

6.5 Discussion

Most of the GDM women who participated in this study were of Asian (approximately 60%) and African/Afro-Caribbean (approximately 25%) origin, however, African/Afro-Caribbean GDM women dominated the obese group (43.1%). In a study, African/Afro-Caribbean women were found more likely to be obese when compared to South Asian and European women, though reasons for high rates of obesity in this group remain to be established (Pomerleau et al. 1999). Though the sample size of the present study was small, however, this disproportionate representation of African/Afro-Caribbean women in high BMI category might have placed them at increased risk of developing GDM. In fact, a recent study showed that the African-American pregnant women had the greatest increased risk of GDM (>76%) as compared to Latinas (58%), Caucasians (46%) and Asians (25%) (Shah et al. 2011). It will also be reasonable to suggest that more extensive and focused studies need to be carried out to explore further this association.

Overweight and obesity during pregnancy are associated with adverse maternal and neonatal outcomes (Huda et al. 2010; Gaillard et al. 2014). Various studies have reported that high pre-pregnancy BMI is associated with increased risks of GDM, neonatal mortality, and obesity in children (Kongubol and Phupong 2011; Johansson et al 2014; Starling et al. 2015). Previous studies found that obesity is associated with alterations in fatty acid levels. A study conducted in Australia among 124 adults showed that the levels of ω-3 PUFA were lower in obese subjects (Micallef et al. 2009). A systematic review based on 21 studies found that overweight or obese adults had lower levels of ω-6 PUFAs (Fekete et al. 2015). Very few studies have evaluated the associations of BMI with fatty acid levels among pregnant women. The study among 129 pregnant women in USA showed that obese pregnant women had lower DHA and AA concentrations as compared to lean pregnant women (Tomedi et al. 2013). Another study shows high level of AA in obese pregnant women (Scifres et al. 2014). Another population based cohort study showed that obese pregnant women had higher AA, total ω-6 PUFA and lower LA concentrations. All ω-3 PUFA concentrations tended to be low in obese pregnant women, particularly α-linolenic acid. In SFAs, 14:0 (myristic acid) was low in obese pregnant women, whereas in MUFAs, obese women had high palmitoleic acid concentrations (Vidakovic et al. 2015). In line with the above-mentioned results, we found that higher BMI was

associated with higher total ω-6 PUFA levels in GDM women. Obese GDM women had higher AA level. All ω-3 PUFA levels tended to be lower among over-weight and obese GDM women, particularly α-linolenic acid. In SFAs, over-weight and obese GDM women had lower 14:0 (myristic acid) levels, whereas in MUFAs, over-weight GDM women had lower 16:1n-7 (palmitoleic acid) concentrations.

From the current study, it is difficult to establish whether BMI causes adverse fatty acids profile, or whether the direction of this association is other way around. It has been shown that higher ω-6 PUFA levels lead to higher adipose tissue development by promoting pre-adipocyte differentiation and that high ω-3 PUFA levels suppresses development of obesity by reducing adipose tissue mass (Buckley and Howe 2009; Muhlhausler and Ailhaud 2013). In the other direction, obesity causes alterations in adipose tissue metabolic and endocrine function leading to an increased release of fatty acids, thus affecting subsequent adiposity and finally creating a vicious cycle (Kishino et al. 2008). Further experimental studies are needed to explore the underlying mechanism.

We conclude that higher pre-pregnancy BMI is associated with higher ω-6 PUFA and lower ω-3 PUFA levels in GDM women. However, we took blood sample once at the time of recruitment (median 28 weeks) and analysed on the basis of pre-pregnancy BMI which could have changed over the course of pregnancy. The timing of these measurements makes it difficult for us to draw conclusions about the direction of any association. Studies with longitudinal measurements of both BMI and fatty acid levels before and during gestation may help to clarify the direction of these association.

CHAPTER 7

Overview, conclusion and future research----------(141-147)

7.1 Overview

The impacts of gestational diabetes mellitus (GDM) on the short and long-term health of women and their offspring are increasingly recognized. In Europe, the estimated prevalence of GDM ranges from 2 to 6% and it is expected to rise with increasing obesity, a strong risk factor for developing the condition. Our research group had previously reported that the levels of docosahexaenoic acid (DHA) and arachidonic acid (AA) are reduced in maternal red blood cell, and foetal red blood cell and plasma phospholipids. DHA and AA are highly unsaturated fatty acids of the omega-3 and omega-6 family, respectively. They are vital structural and functional components of cellular and sub-cellular membranes as well as precursors of diverse bioactive compounds.

The importance of DHA and AA in foetal life has been extensively documented. Of these two fatty acids, DHA is considered to be the most limiting nutrient in pregnancy and lactation because it is scarce or absent in land animal and plant food sources. Moreover, its synthesis from the parent compound -linolenic acid is inefficient. In normal pregnancy, DHA is preferentially transferred by placental selection from maternal to foetal circulation. However, recent studies have reported that placental uptake and transfer of DHA is impaired in pregnancy complicated by GDM.

One of the earliest abnormalities observed in diabetes is the involvement of oxidative stress. Foetuses from mothers with gestational diabetes are at increased risk of developing foetal macrosomia, platelet hyperaggregability and oxidative stress. High blood glucose levels in these new-borns induce the oxidative stress, which, in turn, provokes the high production of highly reactive oxygen radicals, being toxic to cells, especially to the plasma membranes where these free radicals interact with the lipid bilayer. Endogenous antioxidant vitamins and enzymes are responsible for the detoxification of deleterious oxygen radicals. GDM adversely affects the antioxidant status. Treatment with antioxidants may prevent or reverse abnormalities associated with increased oxidative stress in GDM. Contradictory studies have reported that dietary ω-3 LCPUFA supplements may prevent or enhance the organic deterioration, caused by excessive oxidative stress in GDM.

This research study investigated whether;

a) GDM compromises plasma omega-3 and omega-6 fatty acid status of pregnant women and their neonates.
b) High pre-pregnancy body mass index alters the plasma fatty acid profile in GDM women.
c) Impaired status of DHA in GDM pregnant women and their neonates can be ameliorated by DHA-enriched supplementation.
d) DHA-enriched formula enhances the plasma antioxidant vitamins status in gestational diabetic women and their neonates.

7.2 Conclusion

7.2.1 Study 1 (chapter 3)

Consistent to the previous findings by our research group, the levels of DHA and AA were not reduced in plasma lipids in GDM women. It could be due to the failure of incorporation the nutrients into red cell phospholipids. Also, the possibility of impaired placental transport could not be ruled out. Moreover, the samples were collected at diagnosis, and it is plausible that the duration of the diabetes was very short to produce an obvious adverse effect on the plasma DHA and AA levels.

A novel finding of the study is that omega-3 LCPUFA supplementation is effective in enhancing the maternal DHA status in GDM women. Furthermore, it showed that the decline in maternal DHA, which occurs in the final stages of gestation and was thought to be a physiological response to pregnancy, can be halted by supplementation. We suggest that the provision of a DHA supplement should be integrated with the antenatal care of pregnant women with gestational diabetes to optimize foetal development and avert maternal DHA depletion in pregnancy.

7.2.2. Study 2 (chapter 4)

Cord plasma fatty acid levels were comparable between the women with and without gestational diabetes. Nevertheless, the trend was for a relative reduction in omega-6 and omega-3 fatty acids in the cord of the GDM women, although some of the values were not statistically significant. The foetus is reliant on the maternal status and placental uptake. Since there was no obvious evidence of dietary insufficiency or reductions in plasma fatty acids, the reduced DHA and AA levels in the cord plasma of

GDM women could be due to impaired placental uptake. Given the importance of these nutrients, this abnormality may have adverse effects on foetal growth and development as well as health in adulthood. There is a need for further studies to understand the underlying mechanism for this abnormality and its developmental and health implications.

This double blind, placebo-controlled, randomized study demonstrated that supplementation of women with GDM is effective in enhancing maternal but not foetal DHA status. The failure of the supplementation to improve the foetal DHA status is of some concern because the offspring of the women with GDM are born with a lower level of the nutrient and the condition is thought to be link with a risk of neuro-developmental deficits. It is plausible that maternal supplementation with more than 600mg of DHA may trickle down to the foetus and mitigate the insufficiency, or an increased duration of maternal supplementation may improve the neonatal DHA status. Regardless, we suggest that babies of gestational diabetic women, particularly those not suckling, similar to the babies who born prematurely require formula-milk fortified with a higher level of DHA.

7.2.3 Study 3 (chapter 5)

Increased oxidative status is associated with gestational diabetes. No study is available on the improvement of antioxidant vitamin status by omega-3 LCPUFA enriched fish oil in GDM women and their neonates. Our study adds another aspect of omega-3 LCPUFA in the allied management of oxidative stress associated with GDM.

In this study, GDM women who were supplemented with omega-3 enriched formula had comparable plasma vitamins E, A and β-carotene levels when compared with their GDM counterparts who received the placebo, at delivery. Similar results were found when cord plasma levels of these micronutrients were compared between the groups. We observed that the antioxidant effects of the omega-3 supplementation are dose and duration dependent. In this study, the antioxidant status did not improve yet not deteriorate in GDM active-treatment group, however, the dose used and the duration of supplementation was much less in our study as compared to those studies which showed improved antioxidant status. Therefore, we can allude that a moderate dose of omega-3 LCPUFA may be recommended in foods to reduce the incidence and complications associated with oxidative stress in diabetic pregnancy.

7.2.4 Study 4 (Chapter 6)

Obesity during pregnancy is associated with adverse pregnancy outcomes. We evaluated the impact of pre-pregnancy body mass index on plasma fatty acid levels in GDM women. We categorised GDM women on the basis of their pre-pregnancy BMI in normal-weight, over-weight and obese group. As compared to normal-weight women, obese women had higher omega-6 and lower omega-3 PUFA levels. We conclude that higher pre-pregnancy BMI is associated with an adverse fatty acid profile in GDM women.

7.3 Limitations of the study

This study has provided some interesting information about the effect of DHA-rich fish oil on plasma fatty acids and antioxidant vitamin status in pregnancy complicated with gestational diabetes, but it had limitations that future studies may need to take into account.

- It was difficult to recruit sufficient number of healthy control subjects and the low number had a negative implication on the outcome of the statistical analyses. In addition, some of the participants (GDM and Healthy Control) opted out of the study or did not take the supplements as instructed by the mid-wives (lack of compliance).
- A significant number of delivery samples were lost because of unanticipated births before the due date, delivery at a different hospital, too busy labour or delivery midwives to collect samples and women moving out of the area without providing contact details.
- Efforts were made to obtain information about a habitual diet of the women using a food diary, we were unable to extract valuable data about their nutritional intake. This was because most of the diaries returned were incomplete.
- Failure to explain and educate family members about the potential benefit of the study. Most of the pregnant women who attend antenatal clinic in Newham Hospital are of Asian ancestry. In Asian community, decisions to participate or not in a study is a family affair.
- The participants were heterogeneous with respect to genetic and possibly dietary backgrounds and duration of the supplementation period.

The aforementioned limitations, most of which were anticipated during the design of the study protocol, would have been reduced or avoided by recruiting additional research mid-wives and a dietitian, frequent home visits and conducting the study in more than one hospital. However, it was not possible to do so because of budgetary constraints.

7.4 Future investigations

As evident from the conclusions of this study, omega-3 LCPUFA (chiefly DHA) has the potential to be an effective and affordable adjunct to the traditional treatment currently recommended for the management of gestational diabetes. However, further studies are required to be conducted to address the following issues;

1. In the current study, a daily dose of 600 mg of DHA supplementation of GDM women did not enhance the foetal plasma DHA level. The failure of the supplementation to improve the level of foetal DHA is of some concern because the offspring of the GDM women are born with a lower level of the nutrient and the condition is thought to be linked with a risk of neuro-developmental deficits. So, a further study would need to explore;

"Could an increased daily dose of DHA-enriched fish oil (more than 600mg) supplementation in women with GDM trickle down to the foetus and mitigate the insufficiency in plasma n-3 LCPUFA status"?

2. It is reported that foetal LCPUFA insufficiency is associated with high incidence of congenital malformations and impaired neurological development. A direct relationship between deficiencies in LCPUFA and congenital malformations need to be determined. At birth, the adverse effects of gestational diabetes on the offspring may not be immediately apparent. Therefore;

"Follow-up studies in childhood and beyond are necessary to determine the long-term effect of gestational diabetes on the offspring."

3. Placental dysfunction and the consequential reduction in the transfer of the fatty acids from GDM mother may explain the abnormality in the neonates. Therefore;

"Further investigations are very important to determine the placental fatty acids and the activity and expression of placental fatty acid binding and transport proteins in gestational diabetic women."

4. Higher omega-6 and lower omega-3 PUFA levels lead to higher adipose tissue development. In the other direction, obesity causes alterations in adipose tissue metabolic and endocrine function leading to an increased release of fatty acids, thus affecting subsequent adiposity and finally creating a vicious cycle Therefore;

"Further experimental studies are needed to explore the underlying mechanism and the direction of this association"

5. As observed in our study, omega-3 LCPUFA supplementation did not deteriorate the anti-oxidative defence system in GDM. It has been shown in many studies that maternal diabetes significantly alters the "total antioxidant status" as demonstrated by decreased non-enzymatic antioxidants (vitamins A and E), anti-oxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GSSG-Red)). In this study, we determined the non-enzymatic antioxidants status in GDM and their neonates. Therefore;

"Further studies targeting gestational diabetic women and their offspring are recommended to further investigate the effect of omega-3 LCPUFA on total antioxidant status. Moreover, the application of the omega-3 LCPUFA as supplements in the management of oxidative stress associated with gestational diabetes need to be explored."

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APPENDICES

(192-202)

Appendix 1. List of instruments.

Gas-liquid chromatography (GLC)	
Product: 1800 series gas chromatography model 8533 (HRGC MEGA 2 Series) with dual flame ionization detector, split/splitless, amplifiers with A 200S auto-sampler.	
Supplier	Fisons Instruments (UK organic sales, Crewe Road, Wythenshaw, Manchester, M23 9BE)
Column	BPX-70 column
Supplier	SGE Europe Ltd (UK)
Length	60 meters
Physical properties	Highly Polar
Material	70% Cyanopropyl Polysilphenylene-siloxane
Type	BPX70
Film Thickness	0.25 μm
Internal Diameter	0.32mm
Operating temperature	150^{o}C – 250^{o}C
Conditioning temperature	250^{o}C for 5 minutes
Hydrogen generator	
Product	Hygen 400
Supplier	Claind Brezza Generators. Jaytee BioSciences LtD. Uk
▪ This hydrogen generator produces up to 600 cc/min of pure dry hydrogen gas using deionised water and electricity.	

High-performance liquid chromatography (HPLC)	
Product: Agilent 1100 series high performance liquid chromatograph system with a quaternary pump connected to an auto sampler, automatic fraction collector, and column thermostat and diode-array detector.	
Diode array detector	
Model	Agilent 1100 Series diode array detector (DAD)
Supplier	Agilent technologies (Agilent technologies Deutchland, Gmbh, Waldbronn analytical division, Waldbronn, Germany
Light source	Deuterium and tungsten lamps
Wavelength range	190-95-nm
Diode width	< 1nm
Auto sampler	
Model	Agilent 1100 series auto sampler
Dimensions	200×345×435mm
Ambient operating temperature	4-40^{o}C
Humidity	95% at 25-40^{o}C
Hypersil gold HPLC column	
Length	250mm
Diameter	4.6mm
Particle size	5µm
Pore size	175Å
pH range	1-11
Supplier	Thermo Scientific, UK

Appendix 2. List of chemicals.

Name	Company	Code
Absolute Ethanol	Hayman Ltd.	64-17-5
Acetic acid	Fisher scientific UK	A/0400/PB08
Acetyl chloride	Acros organic	151270010
Butylated hydroxytoluene (BHT)	Sigma-Aldrich Co.UK	B-1378
Chloroform, HPLC grade	Fisher scientific UK	C/4966/17
CPG standard	Sigma-Aldrich Co.UK	P5394
2, 7-dichlorofluorescein	Sigma-Aldrich Co.UK	410217
Dichloromethane (DCM)	Fisher scientific UK	D143SK-4
Diethyl ether	Fisher scientific UK	D/2450/17
Dried methanol	Fisher scientific UK	M/4050/15
FAME mix	Sigma-Aldrich Co.UK	18919-IAMP
Formic acid, 98/100%	VWR international	101155F
Heptane	Fisher scientific UK	H/0106/17
Hexane	Sigma-Aldrich Co.UK	H/0406/17
Methanol HPLC grade	Fisher scientific UK	M/4056/17
Methylamine (Extra pure, 40 wt. %)	Acros organic	126230010
Petroleum ether 60-80 degree	Fisher scientific UK	P/1800/17
PBS 10X (Dulbecco)	Sigma-Aldrich Co.UK	D1408
Potassium bicarbonate	Fisher scientific UK	P/5120/53
Sodium chloride	Sigma-Aldrich Co.UK	S 7653
Sodium sulphate (Granular, anhydrous)	Fisher scientific UK	S/6600/53

Thin layer chromatography plates	
Product	TLC silica gel
Cat No.	1.5721.0001
Size	20×20 cm
Layer thickness	210-270 µm
Pore volume	0.74-0.84 m/g
Supplier	Merck KGaA, Germany

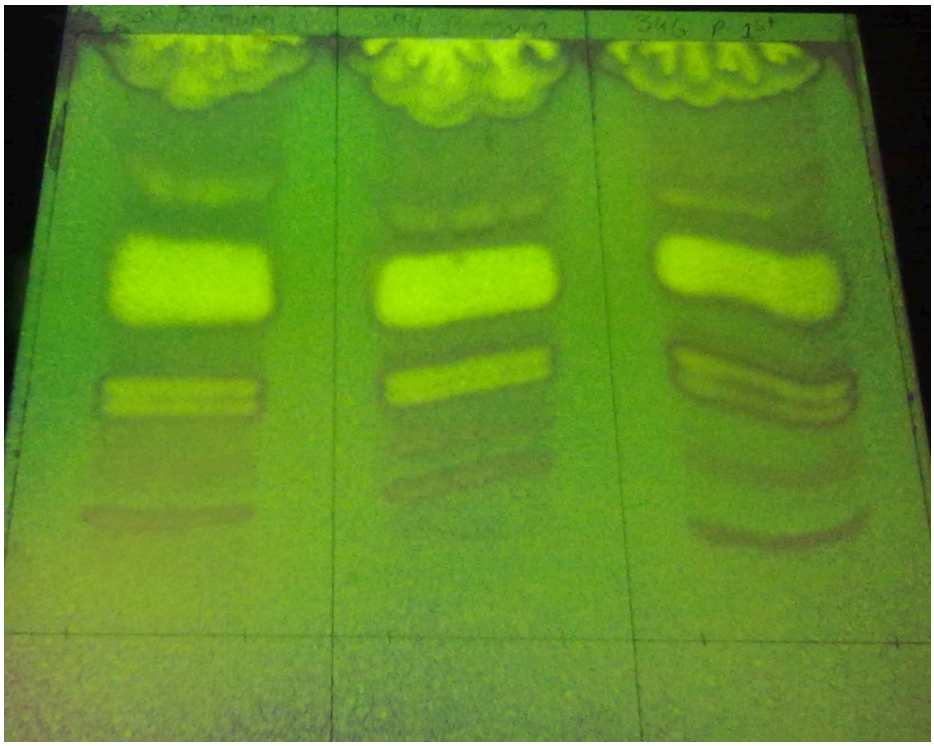
Appendix 3. Fatty acids standard mix: 100 mg ampule contains the following with weight percentages indicated.

Trivial Names	Abbreviation	Weight(%)
Butyric acid methyl ester	4:0	4
Caproic acid methyl ester	6:0	4
Caprylic acid methyl ester	8:0	4
Undecanoic acid methyl ester	10:0	2
Lauric acid methyl ester	12:0	4
Tridecanoic acid methyl ester	13:0	2
Myristic acid methyl ester	14:0	4
Myristoleic acid methyl ester	14:1	2
Pentadecanoic acid methyl ester	15:0	2
Cis-10-Pentadecenoic acid methyl ester	15:1	2
Palmitic acid methyl ester	16:0	6
Palmitoleic acid methyl ester	16:1	2
Heptadecanoic acid methyl ester	17:0	2
Cis-10-Heptadecenoic acid methyl ester	17:1	4
Stearic acid methyl ester	18:0	4
Oleic acid methyl ester	18:1	4
Elaidic acid methyl ester	18:1	2
Linoleic acid methyl ester	18:2	2
Linolelaidic acid methyl ester	18:2	2
α-Linolenic acid methyl ester	18:3	2
γ-Linolenic acid methyl ester	18:3	2
Arachidic acid methyl ester	20:0	4
Cis-11-eicosenoic acid methyl ester	20:1	2
Cis-11,14-eicosadienoic acid methyl ester	20:2	2
Cis-11,14,17-eicosatrienoic acid methyl ester	20:3	2
Arachidonic acid methyl ester	20:4	2
Cis-5,8,11,14,17-eicosapentaenoic acid methyl ester	20:5	2
Heneicosanoic acid methyl ester	21:0	2

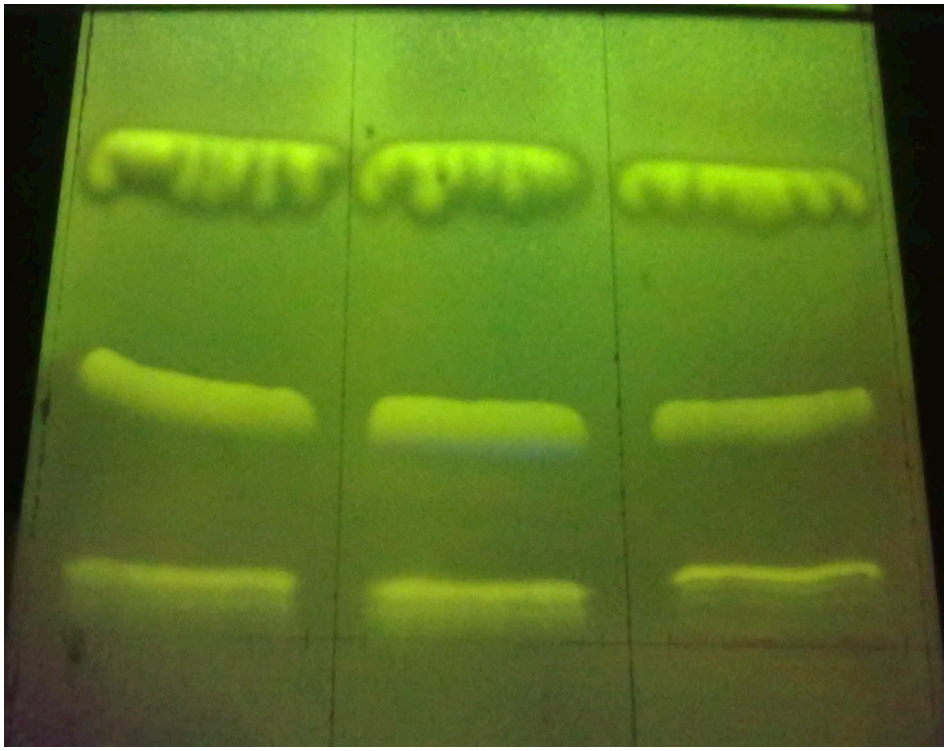
Behenic acid methyl ester	22:0	4
Erucic acid methyl ester	22:1	2
Cis-13,16-docosadienoic acid methyl ester	22:2	2
Cis-7,10,13,16-docosatetraenoic acid methyl ester	22:4	2
Cis-7,10,13,16,19-docosapentaenoic acid methyl ester	22:5	2
Cis-4,7,10,13,16,19-docosahexaenoic acid methyl	22:6	2
ester	23:0	2
Tricosanoic acid methyl ester	24:0	4
Lignoceric acid methyl ester	24:1	2
Nervonic acid methyl ester		

Appendix 4. Vitamins standards.

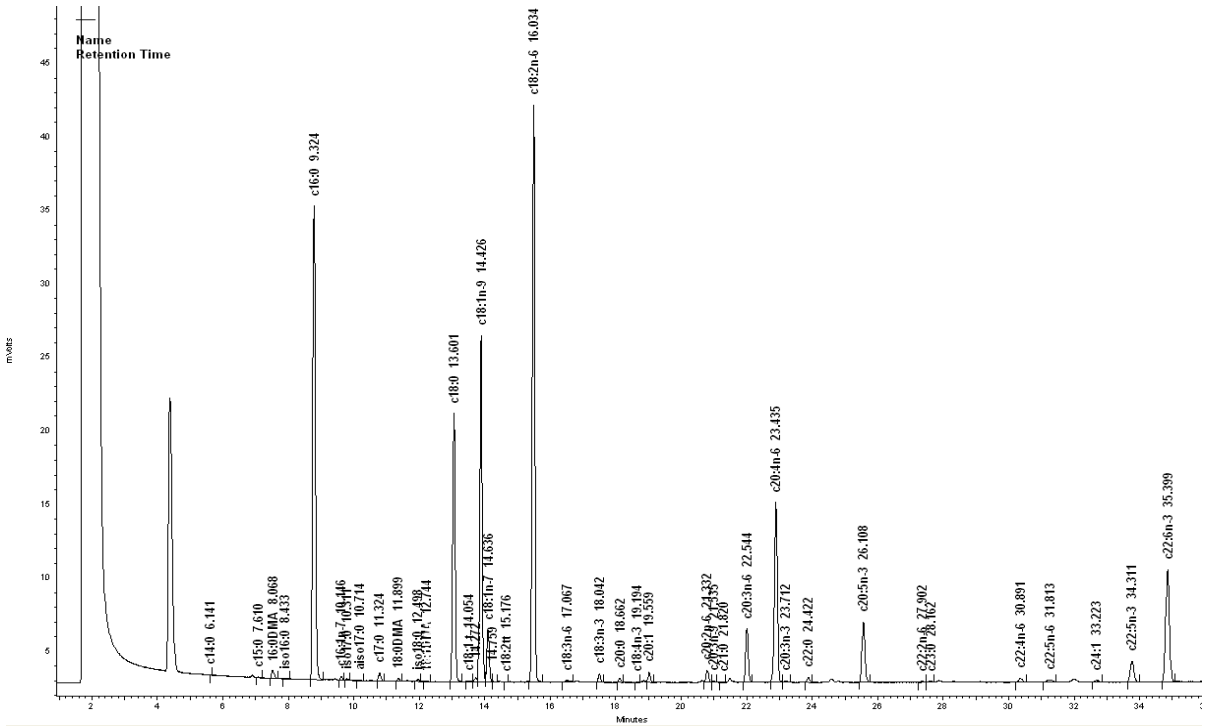
α-tocopherol, purity > 97.0% (HPLC)	Fluka	95240
Retinol, purity > 99.0% (HPLC)	Fluka	95144
β-carotene, purity> 95.0% (HPLC)	Sigma-Aldrich	C4582



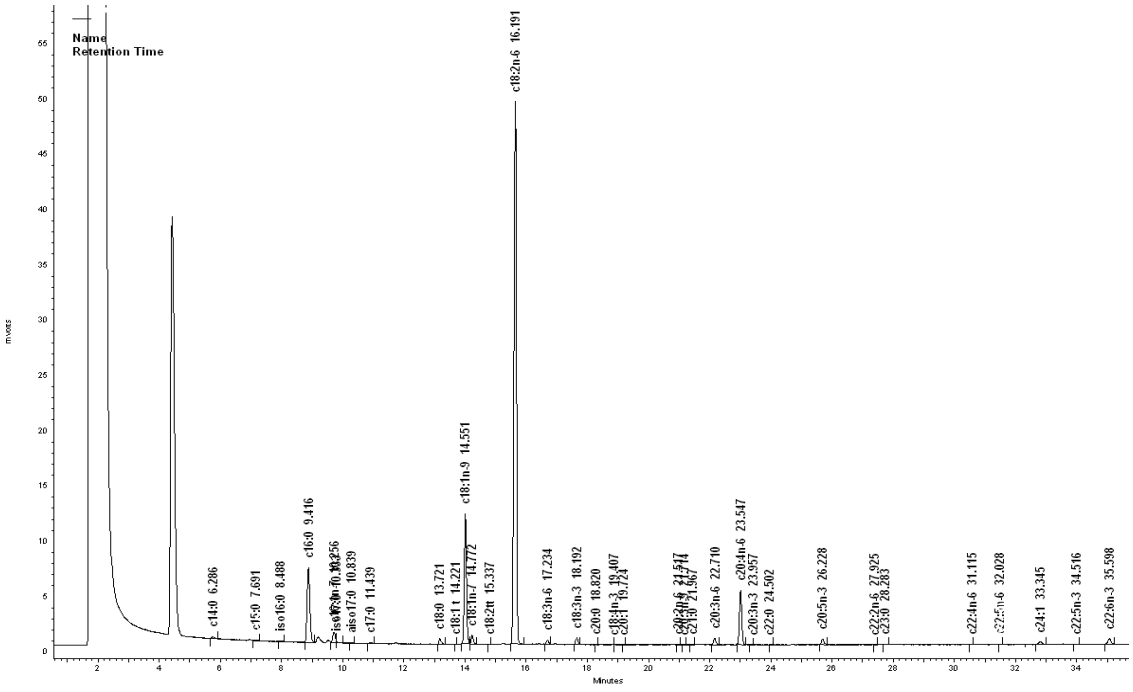
Appendix 5. Thin layer chromatography separation of plasma phospholipids on silica gel plate by single vertical development. Original sample applied at the base point. (The sequence of the bands from top to down is: 1= Neutral lipids, 2= Choline phosphoglycerides, 3= Sphingomyelin).



Appendix 6. Thin layer chromatography separation of plasma neutral lipids on silica gel plate by single vertical development. Original sample applied at the base point. (The sequence of the bands from top to down is: 1= Cholesterol esters, 2= Triglycerides, 3= Free fatty acids).



Appendix 7. A specimen chromatogram of plasma fatty acid methyl esters (CPG fraction) of a test sample.



Appendix 8. A specimen chromatogram of plasma fatty acid methyl esters (CE fraction) of a test sample.